University of Alberta

Peripartum Performance and Amino Acid Metabolism of the Dairy Cow

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

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ABSTRACT

Studies were conducted on multiparous dairy cows to examine effects of prepartum energy and protein intake on periparturient metabolism and lactation performance, and to determine the requirements for, and the efficiency of use of, the amino acids. In the first study, two levels of energy and two levels of protein were tested in a factorial arrangement. Treatments were administered for 21 days prepartum. Increased nutrient density did not affect prepartum feed intake, but postpartum intake was higher for cows fed the high energy diets. Milk and milk component yields were unaffected by treatment. Cows fed the high energy diets had lower plasma non-esterified fatty acid and hepatic triglyceride concentrations and higher acetyl-CoA carboxylase and fatty acid synthase abundance than cows fed the low energy diets. Increased protein intake prepartum resulted in elevated plasma β-hydroxybutyrate concentrations postpartum. The plasma concentration of 3-methylhistidine was lower with the high protein treatments, suggesting lesser amounts of muscle protein degradation compared to the low protein treatments. Plasma amino acid concentrations decreased at calving, with increased concentrations at day 21 except for histidine, methionine, phenylalanine and glutamine. Overall, increased energy density of the prepartum diets had beneficial effects on feed intake and lipid metabolism, but did not improve lactation performance. Increased protein density was not advantageous to performance. Because there is a paucity of data on amino acid metabolism around calving, the second study examined plasma amino acid concentrations during this period. Amino acid concentrations decreased at calving and then increased to prepartum levels within the first few weeks of lactation, except for histidine, methionine, glutamine and glutamate. The third study

evaluated milk protein synthesis relative to amino acid supply. Using data from published studies, regression equations were generated that predicted milk protein yield as a function of duodenal amino acid supply and dietary energy intake. Efficiency of amino acid use for milk protein synthesis declined with increasing milk protein yield. Essential amino acid requirements as a percentage of metabolizable protein were suggested. Values for methionine and lysine were 2.2% and 7.3%, respectively. Published values, which exist for these two amino acids, corroborate our suggested requirements.

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TABLE OF CONTENTS

CHAPTER 1 Review of the Literature ***1***

1.1 Introduction **1**

1.2 Hormonal status of the periparturient cow **2**

1.3 Peripartum dry matter intake **4**

1.4 Periparturient lipid metabolism **6**

1.4.1 Homeorhetic adaptations in lipid metabolism 6

1.4.2 Etiology of fatty liver 8

1.4.3 The fatty liver-ketosis complex 12

1.4.4 Lipogenic enzymes 15

1.4.4.1 acetyl-CoA carboxylase 15

1.4.4.2 fatty acid synthase 16

1.4.4.3 lipoprotein lipase 17

1.5 Periparturient protein metabolism **18**

1.6 Nutritional needs of the dry cow **20**

1.6.1 Nutrients required for conceptus growth 20

1.6.2 Nutrient recommendations for the dry cow 21

1.7 Body tissue mobilization **24**

1.8 Effect of prepartum nutrient intake on the periparturient cow **27**

1.8.1 Energy intake 27

1.8.2 Protein intake 29

1.9 Methods to determine amino acid requirements **32**

1.9.1 Nitrogen balance and milk production response 33

1.9.2 Plasma amino acids 34

1.9.3 Plasma urea 36

1.9.4 Amino acid oxidation 36

1.9.5 Amino acid extraction and transfer efficiency 37

1.10 Current systems for predicting protein requirements of dairy cattle **38**

1.11 Splanchnic modulation of amino acid supply **40**

1.12 Amino acid metabolism in the mammary gland **46**

1.12.1 Protein requirements for lactation 46

1.12.2 Amino acid uptake by the mammary gland 47

1.12.3 Protein turnover in the mammary gland 49

1.12.4 Limiting amino acids for milk protein synthesis 51

1.13 Conclusion **53**

1.14 Objectives **54**

1.15 References Cited **58**

CHAPTER 2 Peripartum Performance and Metabolism of Multiparous Dairy Cows in Response to Prepartum Energy and Protein Intake

2.1 Introduction **69**

2.2 Materials and Methods **70**
2.2.1 Animals and Treatments 70
2.2.2 Sampling and Analysis 73
2.2.3 Statistical Analysis 77
2.3 Results **78**
2.3.1 Metabolic Parameters 78
2.3.2 Lipid Metabolism 79
2.3.3 Protein Metabolism 81
2.3.4 DMI, BW, BCS, and Energy and Protein Balance 82
2.3.5 Milk Yield and Composition 84
2.3.6 Nutrient Digestibility 85
2.4 Discussion **85**
2.4.1 Lipid Metabolism and Metabolic Parameters 85
2.4.2 Protein Metabolism 91
2.4.3 DMI, BW, BCS, and Energy and Protein Balance 94
2.4.4 Milk yield and composition 95
2.4.5 Nutrient Digestibility 96
2.5 Conclusion **97**
2.6 References Cited **121**

CHAPTER 3 Plasma Amino Acid Concentrations During the Periparturient Period

3.1 Introduction **126**
3.2 Materials and Methods **127**
3.2.1 Diets and sampling 127
3.2.2 Statistical analysis 128
3.3 Results **129**
3.4 Discussion **130**
3.5 Conclusion **134**
3.6 References Cited **140**

CHAPTER 4 Evaluation of Milk Protein Synthesis as a Function of Amino Acid Supply

4.1 Introduction **142**
4.2 Materials and Methods **144**
4.2.1 Source of data 144
4.2.2 Data analysis 145
4.3 Results **149**
4.3.1 Milk protein yield 149
4.3.2 Milk protein yield response 151
4.3.3 Efficiency of amino acid use 152
4.4 Discussion **153**

4.4.1 Protein yield 153
4.4.2 Protein yield response 156
4.4.3 Efficiency of amino acid use 158
4.4.4 Limitations to this approach 164
4.5 Conclusion 168
4.6 References Cited 179

CHAPTER 5 General Discussion and Conclusions 185
5.1 Conclusions 185
5.2 General Discussion 187
5.3 Future Perspectives 191
5.4 References Cited 193

Appendix A: Analytical Procedures 195
A.1. Liver biopsy procedure 195
A.2. Adipose tissue biopsy 195
A.3. Nonesterified fatty acid microassay 196
A.4. D-ß-Hydroxybutyrate assay (BHBA) 198
A.5. Insulin assay 199
A.6. IGF-1 assay 201
A.7. Growth hormone assay 203
A.8. 3-Methylhistidine analysis by HPLC 205
A.9. Amino acid analysis by HPLC 207
A.10. Liver lipid extraction 209
A.11. Triglyceride analysis from liver lipid extract 211
A.12. Liver glycogen assay 213
A.13. Chromium assay 215
A.14. Cytosolic protein isolation 217
A.15. Quantitation of protein 219
A.16. Western blotting 220
A.17. Acetyl-CoA carboxylase activity assay 226
A.18. Fatty acid synthase activity assay 228
A.19. Lipoprotein lipase activity assay 230

Appendix B: Description of publications used to generate the amino acid database described in chapter 4 234

LIST OF TABLES

Table 1-1 CNCPS (2000) efficiency values for absorbed amino acid use. 56

Table 2-1 Ingredient and nutrient composition of prepartum dietary treatments..... 98

Table 2-2 Ingredient and nutrient composition of the postpartum diet (DM basis)..... 99

Table 2-3 Adipose tissue acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) abundance around the time of calving 100

Table 2-4 Adipose tissue acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and lipoprotein lipase (LPL) activity around the time of calving 101

Table 2-5 Plasma amino acid concentrations (μM) around the time of calving. 102

Table 2-6 Effect of prepartum dietary treatment on lactation performance and change in BW and BCS. 103

Table 2-7 Milk fatty acid composition at 2 and 6 wk postpartum. 104

Table 2-8 Digestibility of nutrients in cows fed different diets prepartum. 105

Table 3-1 Ingredient and nutrient composition of the TMR and hay..... 135

Table 3-2 Plasma amino acid concentrations (μM) around the time of calving. 136

Table 4-1 Dry matter intake and production of cows from all studies in the database. 169

Table 4-2 Dry matter intake and production of cows from studies used in the protein yield response analyses. 170

Table 4-3 Amino acid profile of whole empty body of cattle. 171

Table 4-4 Prediction equations for milk true protein yield (PY). 172

Table 4-5 Prediction equations for milk true protein yield response (PYRPCT). 173

Table 4-6 Efficiency of use of infused amino acids 174

Table 4-7 Efficiency of amino acid use for lactation. 175

Table 4-8 Requirement for individual amino acids (% of protein) in lactating dairy cows. 176

Table 4-9 NRC (2001) predicted duodenal amino acid flows vs. actual flows. 177

LIST OF FIGURES

Figure 1-1 Two-phase broken line plasma amino acid response curve. 57

Figure 2-1 Plasma ß-hydroxybutyrate (BHBA) concentrations around the time of calving 106

Figure 2-2 Plasma glucose concentrations around the time of calving 107

Figure 2-3 Plasma insulin concentrations around the time of calving 108

Figure 2-4 Plasma IGF-1 and growth hormone (GH) concentrations around the time of calving 109

Figure 2-5 Plasma nonesterified fatty acid (NEFA) concentrations around the time of calving 110

Figure 2-6 Concentrations of liver triglyceride (upper panel) and glycogen (lower panel) (DM basis) of cows during the transition period. 111

Figure 2-7 Western blot of ACC protein (265 kDa, upper panel) and FAS protein (260 kDa, lower panel) in adipose tissue. 112

Figure 2-8 Plasma urea nitrogen (PUN) concentrations around the time of calving 113

Figure 2-9 Plasma 3-methylhistidine (3-MH) concentrations around the time of calving. 114

Figure 2-10 Urinary 3-methylhistidine (3-MH):creatinine ratio around the time of calving. 115

Figure 2-11 Daily DMI during the periparturient period 116

Figure 2-12 Net energy balance during the periparturient period 117

Figure 2-13 Crude protein balance during the periparturient period 118

Figure 2-14 Bodyweight (BW) around the time of calving. 119

Figure 2-15 Milk yield during the first 6 wk postpartum 120

Figure 3-1 Daily dry matter intake (DMI) around the time of calving 137

Figure 3-2 Body weight (BW) around the time of calving 138

Figure 3-3 Daily milk yield during the first 7 wk of lactation 139

Figure 4-1 Milk true protein yield (PY) vs. amino acid supply. 177

LIST OF ABBREVIATIONS

AA – amino acid
AAFM – AA available for milk
AAI – digestible duodenal AA flow
AAS – AA supply from infusion
AAT – AAI + AAS
ACC – acetyl-CoA carboxylase
ADF – acid detergent fibre
AP – absorbed protein
ARG - arginine
BCAA – branched-chain amino acid
BCS – body condition score
BHBA – ß-hydroxybutyrate
BW – body weight
CP – crude protein
CPI – dietary CP intake
CPT – CPI + CP supplied from infusion
DIM – days in milk
DM – dry matter
DMI - dry matter intake
EAA – essential amino acid
FA – fatty acid
FAS – fatty acid synthase
GH – growth hormone
GLN - glutamine
GLU – glutamate
HEHP – high energy, high protein
HELP – high energy, low protein
HIS - histidine
HSL – hormone sensitive lipase
IGF-1 – insulin-like growth factor-1
ILE – isoleucine
LEHP – low energy, high protein
LELP – low energy, low protein
LEU - leucine
LPL – lipoprotein lipase
LYS – lysine
MDV – mesenteric drained viscera
MET – methionine
MP – metabolizable protein
MPI – dietary MP intake
MPIR - MPI/MP requirement
MPR – MP requirement
MPS – MP supplied from infusion

MPT – MPI + MPS
NDF – neutral detergent fibre
NEAA – nonessential amino acid
NEFA – nonesterified fatty acid
NE_L – net energy of lactation
NELI – NE_L intake derived from diet
NELT – NE_L intake derived from diet + infusion
NFC – nonfibre carbohydrate
NRC – National Research Council
OM – organic matter
PDV – portal drained viscera
PHE – phenylalanine
PUN – plasma urea nitrogen
PY – true milk protein yield
PYR - true milk protein yield response
PYRPCT – PYR as a % of control milk yield
RDP – rumen degradable protein
RUP – rumen undegradable protein
TG - triglyceride
THR – threonine
TMR – total mixed ration
TRP - tryptophan
3-MH – 3-methylhistidine
VAL - valine
VLDL – very low density lipoprotein

CHAPTER 1

Review of the Literature

1.1 Introduction

The transition period for the dairy cow, defined as 3 wk before calving to 3 wk after calving (Grummer, 1995), is a period of metabolic upheaval. During this time the dairy cow's metabolic priorities for available nutrients change from net tissue deposition to secretion of large quantities of milk frequently requiring tissue mobilization. This metabolic transition begins several weeks prepartum and involves altered nutrient metabolism in adipose tissue, liver, skeletal muscle, and mammary gland with endocrine changes that are involved in initiation of parturition and lactogenesis. Another common feature of the transition period is a decline in dry matter intake (DMI) during the last two to three weeks precalving (Bertics et al., 1992). This reduction in DMI occurs at a time when nutrient demands are increasing. Disturbances in immunological function also occur during this time (Goff and Horst, 1997), and in conjunction with the drop in feed intake, make the cow prone to metabolic disorders.

Attempts have been made to improve postpartum productive performance of the cow by manipulating dietary energy and/or protein intake prepartum. The studies that have examined protein intake have involved either increasing the crude protein (CP) content of the diet, or changing the ratio of rumen degradable protein (RDP) to rumen undegradable protein (RUP). There have been very few studies in which the profile of amino acid (AA) supply has been examined; consequently, the AA requirements of the transition cow are unknown. Similarly, the AA requirements of the lactating cow are not yet well defined. Determination of the AA requirements is considerably more difficult in

ruminants than in non-ruminants. The large variety of feed ingredients fed to ruminants combined with the equally large variation in ingredient composition, plus the alterations in dietary protein AA profile as a result of rumen metabolism makes prediction of duodenal AA supply challenging. Furthermore, intestinal and hepatic use of intestinally delivered AA will also affect the quantity and profile of amino acids supplied to the mammary gland.

This chapter provides a review of the physiological and metabolic status of the multiparous dairy cow during the transition period, and discusses nutritional strategies to smooth the transition from gestation to lactation. The chapter then focuses on protein nutrition in the lactating cow, with special attention on AA metabolism. The metabolism of AA both in the splanchnic tissues (portal-drained viscera and liver) and mammary gland is discussed, as are some of the approaches used to determine AA requirements.

1.2 Hormonal status of the periparturient cow

The last month before calving is a time of homeorhetic adaptation for the dairy cow, involving changes in concentration of several hormones. Plasma progesterone concentration is high during pregnancy, peaking at 7 to 8 ng/ml approximately one month before calving (Goff and Horst, 1997). Progesterone is necessary for maintenance of pregnancy, by depressing myometrial contractility, and inhibition of lactose synthesis, which prevents the initiation of milk production. Beyond 250 d of gestation, progesterone levels fall to 3 to 4 ng/ml and remain at this level until the day before calving, at which time the concentration falls to nearly undetectable levels. Plasma estrogen, which is quite low during early pregnancy, increases during mid-gestation up to ~300 pg/ml. At about the time that progesterone levels begin to decline, estrogen

concentrations rise again reaching levels of ~2000 pg/ml one week before calving and 4000 to 6000 pg/ml immediately before calving (Goff and Horst, 1997). The rise in estrogen concentration in late pregnancy overcomes the progesterone block of myometrial contractility (Reeves, 1987). Estrogen also increases the amplitude and frequency of uterine contractions by potentiating the effects of oxytocin and prostaglandin $F_2\alpha$ (Reeves, 1987). The change in relative concentrations of estrogen and progesterone that starts at about 30 d prepartum is initiated by the fetus (Goff and Horst, 1997).

Plasma cortisol concentrations increase three to four-fold during the 3 d before parturition (Goff and Horst, 1997). Cortisol stimulates gluconeogenesis and lipolysis, ultimately yielding glucose, which is required at parturition for uterine contraction and expulsion of the calf. Prolactin, which is involved in the initiation of galactopoiesis, also rises in concentration substantially 1 d before calving (Goff and Horst, 1997). Both hormones fall to near prepartum levels the day after calving (Edgerton and Hafs, 1973).

Insulin levels decline prior to parturition (Gerloff et al., 1986) while growth hormone (GH) concentrations rise (Kunz et al., 1985). This change in hormonal profile ensures that nutrients will be directed to the mammary gland thus promoting survival of the offspring, which is the metabolic priority of the dam. Insulin-like growth factor-1 (IGF-1) concentration falls during the immediate prepartum period (VandeHaar et al., 1999). Uncoupling of GH and IGF-1 occurs when cows are in negative energy balance (McGuire et al., 1995).

1.3 Peripartum dry matter intake

Dry matter intake of the multiparous dairy cow typically declines 30% during the last three weeks of gestation (Bertics et al., 1992; Van Saun et al., 1993; VandeHaar et al., 1999). Multiple factors contribute to the reduction in intake, the first of which may be physical limitations. As the fetus grows and the uterus takes up an increasing amount of space in the abdominal cavity, the space available for the rumen declines and thus may restrict intake. The possibility of a physical limitation to intake was examined by Bertics et al. (1992). In their study, cows were force-fed through the rumen cannula for the last 17 d prepartum. During this time intake could be maintained at the same level as was measured during d 21 to 17 before calving, suggesting that rumen capacity is not a limitation to prepartum DMI and that endocrine factors may be involved.

One of the endocrine factors that may contribute to the decline in feed intake before calving is the prepartum peak in plasma estradiol levels. Cows that were treated with estradiol-17β experienced a reduction in DMI compared to control cows (Grummer et al., 1990). However, administration of a single dose of estradiol-17β to cows on d 276 of gestation did not influence prepartum intake but tended to reduce postpartum DMI (Bremmer et al., 1999).

Opioid peptide concentrations, such as that of β-endorphin, increase during the last month prepartum. It is possible that because of their ability to decrease gastrointestinal motility, opioids contribute to the reduction in feed intake prepartum (Goff and Horst, 1997).

Energy content of the diet may also influence the precalving reduction in DMI. Coppock et al. (1972) fed 4 diets ranging from 75% to 30% forage (DM basis) and found

that the percentage decrease in feed intake in the immediate prepartum period was greatest for cows consuming the diet with the least forage. Johnson and Otterby (1981) also observed declines in DMI with high concentrate diets. In their study, cows on a high concentrate diet had higher DMI from d -28 to d -13 compared to cows on a low concentrate diet. Thus, even though their drop in intake was greatest from d –12 to calving, their DMI at calving was equivalent to cows on the low concentrate diets and their energy intake was higher. Hernandez-Urdaneta et al. (1976) also found that feed intake depression was greater for cows consuming diets with 20% concentrate as opposed to those containing 5% concentrate, but energy intake was still higher in the cows on the 20% concentrate diet. Other researchers have not reported an accelerated reduction in intake with high energy diets. VandeHaar et al. (1999) fed diets ranging from 8% to 53% concentrate and found no difference in intake during the last two weeks before calving. In the study of Minor et al. (1998), prepartum DMI of a diet containing 56% concentrate was higher than that of a diet containing 13% concentrate. In general, it appears that cows consuming high concentrate diets in the prepartum period will maintain higher energy intakes even if the rate of decrease in feed intake is accelerated compared to cows consuming low concentrate diets.

Body condition of the cow also influences prepartum feed intake. Emery (1993) examined the feed intake of 20 cows in the dry period. Cows with the highest body condition score (BCS > 3.6) consumed dry matter at 1.5% of body weight (BW) and cows with the lowest body condition score (BCS < 3.6) consumed dry matter at 2% of BW. Similarly, Hartwell et al. (2000) found that DMI of dry cows with BCS of 3.3 was 12.6 kg whereas DMI of cows with BCS of 3.8 was 11 kg. A similar feed intake

response to BCS is seen postpartum. Holter et al. (1990) and Garnsworthy and Jones (1987) reported that cows that were overconditioned at calving had poor appetites postpartum compared to thinner cows. Grummer (1995), using feed intake data from three studies involving 40 cows, found that there was a negative correlation between prepartum BCS and feed intake at 21 d postpartum ($r = -0.45$, $P < 0.006$).

Maximizing DMI in the periparturient period is essential for the cow to have a successful transition from gestation to lactation. Feed intake in the prepartum period appears to be correlated with DMI in the postpartum period. Grummer (1995) reported that intake on the day before calving was correlated to intake on d 21 after calving ($r = 0.54$, $P < 0.001$). Dewhurst et al. (2000) also found significant correlations between DMI at wk 2 and 3 before calving and DMI at wk 2 to 7 after calving.

1.4 Periparturient lipid metabolism

1.4.1 Homeorhetic adaptations in lipid metabolism

Mid-gestation is characterized by replenishment of body lipid reserves. This is in large part regulated by the energy and endocrine status of the animal. When cows are in positive energy balance, as occurs after peak milk production, plasma insulin levels are high. Insulin is a lipogenic hormone stimulating triglyceride (TG) formation (Voet and Voet, 1995). Insulin decreases cyclic AMP (cAMP) levels, leading to dephosphorylation and inactivation of hormone sensitive lipase (HSL), thereby inhibiting lipolysis. At the same time, insulin dephosphorylates acetyl-CoA carboxylase (ACC), thereby activating it. This promotes lipogenesis concurrent with the inhibition of lipolysis.

During very late gestation, the dairy cow experiences a shift in tissue metabolism from anabolism to catabolism. This occurs as a result of the increasing needs of the fetus

and the homeorhetic controls the fetus transmits to the dam (Bauman and Currie, 1980). The depression in feed intake that occurs during the last two to three weeks of gestation also contributes to this metabolic shift (Grummer, 1995). To support the needs of the conceptus at this time, the dam must utilize tissue reserves. This is achieved through alterations in the rates of lipogenesis, esterification, and lipolysis. Lipogenesis decreases 35 to 50% from 4 to 2 weeks prepartum, and continues to decrease during the postpartum period. Lipogenesis rates at one month postpartum are only 7 to 10% of one-month prepartum rates (McNamara and Hillers, 1986a, 1986b). Basal lipolysis rates are 10% greater at 1 month prepartum than at 2 wk after dry-off (McNamara and Hillers, 1986c). Catecholamine-stimulated lipolysis is also enhanced at this time, probably mediated through the effects of GH (Bauman, 1976). Epinephrine and norepinephrine bind to β-adrenergic receptors, eliciting an increase in cAMP. Cyclic AMP activates cAMP-dependent protein kinase, which then phosphorylates HSL. Phosphorylation activates HSL, thus stimulating lipolysis in adipose tissue. The increase in lipolysis is evidenced by increases in plasma nonesterified fatty acids (NEFA) and glycerol, and by in vitro NEFA and glycerol release and HSL activity (McNamara, 1991).

The increase in lipolysis and the decrease in lipogenesis before the energy demands of lactation begin suggest that these activities are hormonally regulated. The peak in estrogen before calving may increase the propensity for fatty acid (FA) mobilization from adipose tissue during late pregnancy independent of any change in feed intake and energy balance. Chronic treatment of nonpregnant ovariectomized ewes with estradiol-17ß to levels equivalent to those of near-term pregnant ewes caused increases in plasma NEFA consistent with those seen during late pregnancy (Bell, 1995). The alterations in adipose

tissue metabolism coincide with the timing of mammary differentiation, which is initiated by prolactin and inhibited by progesterone (McNamara and Hillers, 1986a). It is quite likely that adipose tissue and the mammary gland are regulated by the same endocrine controls.

Insulin and GH are also involved in prepartum adipose tissue adaptation. As parturition nears, insulin concentrations fall and GH levels rise. One of the mechanisms by which lipogenesis is depressed is through a reduction in activity of ACC, the key regulatory enzyme in the FA synthesis pathway. Growth hormone impairs insulin's ability to activate ACC, rendering adipose tissue insensitive to the effects of insulin (Vernon et al., 1991). When multiparous Holstein cows are treated with GH, a reduction in lipogenesis occurs with a decrease in ACC activity of 88% (Lanna et al., 1995). Prolactin may also have a role in insulin resistance. In the lactating rat, prolactin appears to inhibit insulin actions in adipose tissue (Williamson and Lund, 1994). The combined effects of low insulin levels and insulin resistance would also promote lipolysis.

Lipolysis rates are increased further postpartum by the high energy demands of lactation, and remain high for at least the first six months postpartum (McNamara and Hillers, 1986a). The NEFA released upon lipolysis enter the blood circulation and can be extracted by various body tissues, including the mammary gland and the liver. If the uptake of NEFA by the liver is high, fatty liver and ketosis may develop.

1.4.2 Etiology of fatty liver

Fatty liver, which refers to the condition of hepatic TG infiltration, occurs when hepatic FA esterification exceeds TG disappearance from the liver. It occurs primarily in periparturient cows, affecting approximately 20 to 70% of dairy cows at calving (Reid,

1980; Emery et al., 1992). Fatty liver is a physiological problem for the cow and an economic problem for the dairy producer because it results in increased culling (Gerloff et al., 1986), decreased reproductive performance (Reid, 1983), impaired gluconeogenesis (Cadórniga-Valiño et al., 1997) and ureagenesis (Strang et al., 1998), accelerated body condition loss, and increased disease incidence (Gerloff et al., 1986). Fatty liver can be classified according to the degree of fat infiltration (determined histologically): mild - 0 to 20% fat; moderate - 20 to 40% fat; and severe - > 40% fat (Reid, 1980). These levels correspond to TG concentrations, by weight, of 0 - 5%, 5 - 10%, and > 10%, respectively.

Fatty liver was commonly thought to begin postpartum when energy intake is substantially less than the energy required for maintenance and milk production. However, it is now well documented that fatty liver results from negative energy balance in the immediate prepartum period (Bertics et al., 1992; Gerloff et al., 1986; Skaar et al., 1989). During this time lipid mobilization from adipose tissue results in increased plasma NEFA concentrations. Bertics et al. (1992) reported a 2.5-fold increase in NEFA between d 17 before calving and d 1 after calving. As NEFA concentrations increase in blood, liver FA uptake increases (Emery et al., 1992), and the extracted NEFA are subject to one of three fates: reesterification, complete oxidation to CO_2, or incomplete oxidation to ketone bodies. Even though the percentage of plasma NEFA oxidized to CO_2 increases as plasma NEFA concentration increases (Pullen et al., 1989), there is an upper limit to hepatic NEFA oxidation. Several hypotheses have been put forward to explain this: 1) when energy, specifically carbohydrate, availability is limiting, there may insufficient oxaloacetate to maintain the TCA cycle due to the increased demand for oxaloacetate for

gluconeogenesis (Baird et al., 1968); 2) a deficiency of niacin, which favors ketogenesis over complete oxidation; 3) a carnitine deficiency, which would reduce mitochondrial transport of FA and thus oxidation; both Drackley et al. (1991) and Jesse et al. (1986) found that carnitine supplementation increased palmitate oxidation; 4) a deficiency of choline; Carter and Frenkel (1978) reported a reduction in carnitine levels and FA oxidation as a result of choline deficiency; and 5) endocrine factors, such as high estrogen concentrations and low progesterone concentrations, which favor esterification over oxidation (Grummer, 1995). Fatty acids that are not oxidized to completion will be partially oxidized to ketone bodies or reesterified, resulting in increased hepatic TG concentrations. There is a strong relationship ($r = 0.6$, $P < 0.001$) between plasma NEFA concentration and liver fat infiltration (Reid et al., 1983).

The rate of FA esterification depends on the supply of FA to the liver. As the concentration of plasma NEFA increases prepartum the rate of esterification increases (Bauchart et al., 1996). The elevation in hepatic esterification may be attributable to endocrine changes associated with parturition. In cows with elevated NEFA concentrations, such as those approaching parturition, estrogen administration increases TG accumulation in the liver (Grummer et al., 1990). Estrogen may only elicit a response when administered continuously to cows in severe negative energy balance. When administered as a single dose to prepartum cows that did not experience large reductions in DMI, estradiol had no effect on plasma NEFA or liver TG content (Bremmer et al., 1999). In non-ruminants, estrogen increases liver FA esterification (Weinstein et al., 1977). Esterification is further enhanced by low progesterone levels. The drop in progesterone that ruminants experience at calving may therefore have an

influence on fatty liver development. Increases in epinephrine or norepinephrine at parturition may contribute to elevated plasma NEFA and liver TG concentrations at calving.

Feed intake depression is an important determinant of the extent of hepatic TG accumulation that occurs by calving. In multiparous cows, gradual reductions in DMI often begin 3 wk prepartum and accelerate during the final 7 d prior to calving (Bertics et al., 1992; VandeHaar et al., 1999; Greenfield et al., 2000). Bertics et al. (1992) reported a 28% decrease in intake in control cows compared to cows force-fed through rumen cannulae for the final 17 days before calving. The hepatic TG content of cows that were force-fed increased 75% between d 17 before calving and d 1 after calving, whereas it increased 227% in cows that were allowed to experience feed intake depression. The correlation between feed intake on d 1 before calving and liver TG concentration on d 1 after calving was –0.8. Overconditioned cows often have greater hepatic TG accumulation than thinner cows (Fronk et al., 1980). Cows with a BCS of 3.8 at 28 d prepartum had greater liver TG content throughout the periparturient period (-28 d to 56 d relative to calving) than cows with a BCS of 3.3 (Hartwell et al., 2000). This is probably a result of lower DMI, leading to increased lipid mobilization and elevated plasma NEFA concentrations.

The slow rate of TG export from the liver as very low density lipoproteins (VLDL) is a major contributor to the development of fatty liver (Bauchart et al., 1996). In ruminants, it appears that the rate of TG export as VLDL does not increase when hepatic FA uptake is high (Gerloff et al., 1986). Armentano et al. (1991) reported that VLDL TG export was similar between hepatocytes isolated from lactating goats in positive or

negative energy balance. There is some evidence indicating that VLDL secretion rate may be reduced in cows with elevated liver TG, further promoting development of fatty liver (Mazur et al., 1989; Reid et al., 1983). However, low plasma VLDL concentrations do not necessarily mean reduced hepatic secretion. There may be enhanced plasma clearance of VLDL to supply the mammary gland with sufficient substrate for lipid synthesis.

There is a relationship between dependence on the liver as a site for lipogenesis and ability of the liver to export VLDL. Species such as fish and poultry, in which the liver is the major site of lipogenesis, have a high rate of TG export (Pullen et al., 1990). Ruminants, in which the liver provides little FA synthesis, have a slower export rate. Deficiencies of choline and methionine (Met) may contribute to this slow rate as both are involved in phospholipid formation and thus lipoprotein formation (Overton and Piepenbrink, 1999).

1.4.3 The fatty liver-ketosis complex

Fatty liver and ketosis are related metabolic disorders. Ketosis is the accumulation in body fluids of the ketone bodies, acetoacetic acid and β-hydroxybutyric acid (BHBA), and their decarboxylation products, acetone and isopropanol (Kronfeld, 1982). Ketosis is characterized by inappetence, rapid loss of body condition, hypoglycemia, and depressed liver glycogen concentrations. Ketosis occurs during periods of increased hepatic FA uptake when there is incomplete oxidation of the FA. Aiello et al. (1984) suggested that the increase in ketogenic activity of the liver in early lactation is a result of the increased activity of carnitine palmitoyltransferase relative to its activity in late lactation.

Ketosis in dairy cows can be classified into 4 types – primary underfeeding, secondary underfeeding, alimentary, and spontaneous (Kronfeld, 1982). Primary underfeeding ketosis occurs when the cow is not offered enough palatable, nutritionally adequate feed. Secondary underfeeding ketosis occurs when the cow consumes insufficient feed as a result of another disease. Alimentary ketosis is the result of the cow consuming excessive amounts of ketogenic feeds. Spontaneous ketosis occurs when the cow is consuming a ration that is nutritionally adequate, and appears to be due to a glucose shortage caused by the heavy demands of milk production in early lactation. Clinical symptoms of spontaneous ketosis appear 10 to 21 d after calving.

Cows with spontaneous ketosis generally have elevated hepatic lipid concentrations prior to the onset of the ketosis (Drackley et al., 1991; Veenhuizen et al., 1991). Cows are most susceptible to ketosis at 2 to 3 wk postpartum while fatty liver is prevalent by d 1 after calving (Foster, 1988; Grummer, 1993). Drackley et al. (1991) induced ketosis by using moderate feed restriction along with administration of 1,3-butanediol, a ketone precursor, and demonstrated that cows that developed ketosis developed fatty liver first. The observed changes in blood metabolites and liver TG concentration occurred at least 2 wk before cows were diagnosed as being ketotic. Fronk et al. (1980) also demonstrated that ketosis occurred secondary to development of fatty liver.

Spontaneous ketosis is dependent not only on a rise in plasma NEFA concentration but also on a simultaneous decrease in available gluconeogenic precursors (Aiello et al., 1984). In early lactation, the increased requirement for glucose depletes hepatic glycogen reserves. The liver TG:glycogen ratio may be an indicator of the cow's susceptibility to ketosis (Drackley et al., 1991). High hepatic lipid content is usually associated with low

hepatic glycogen content. Bertics et al. (1992) reported that liver glycogen was negatively correlated to liver TG content at 28 d postpartum and that there was a significant positive correlation between the liver TG:glycogen ratio and plasma BHBA concentration. The liver TG:glycogen ratio may indicate the relative supply of lipogenic and glucogenic precursors. Kronfeld (1982) hypothesized that availability of lipogenic precursors for milk production relative to glucogenic precursors determines the cow's susceptibility to spontaneous ketosis.

As with fatty liver, periparturient DMI has a profound effect on the development of ketosis. Cows that were force-fed prepartum had lower levels of plasma BHBA at 14 d postpartum than cows that experienced a drop in DMI (Bertics et al., 1992). Bahaa et al. (1997) reported that limiting feed intake to 50% of the day's previous intake at d 5 after calving resulted in ketosis in 80% of the cows.

Body condition score of the cows also exerts an effect on the incidence of ketosis, with overconditioned cows being at greater risk. Fronk et al. (1980) reported that overconditioned cows have a higher plasma BHBA concentration than thinner cows, as well as elevated plasma NEFA. Overconditioned cows also have lower DMI postpartum (Fronk, 1980), have greater stores of body fat, and more readily develop insulin resistance. These factors allow greater mobilization of fat from adipose tissue and enhanced hepatic fat deposition. In the study of Tesfa et al. (1999) cows with BCS > 4 at 6 wk before calving lost more condition by 6 wk after calving than cows with BCS < 4. The authors also noted that there was a positive correlation with prepartum BCS and postpartum hepatic lipid infiltration. Correa et al. (1990), using data from 32 dairy herds,

concluded that feeding cows to gain weight during the dry period increased the risk of developing ketosis.

1.4.4 Lipogenic enzymes

Lipogenesis in ruminants occurs primarily in adipose tissue. Unlike non-ruminants, there is virtually no lipogenesis in the liver due to the absence of hepatic ACC. Three key enzymes involved in adipose tissue lipogenesis are ACC, fatty acid synthase (FAS), and lipoprotein lipase (LPL).

1.4.4.1 acetyl-CoA carboxylase

Acetyl-CoA carboxylase, a biotin-dependent enzyme, catalyzes the first committed step of FA biosynthesis, which is the conversion of acetyl-CoA to malonyl-CoA. This is a 2-step process involving CO_2 activation and a carboxylation:

1. ACC-biotin + HCO_3- + ATP $\longrightarrow$ ACC-biotin-CO_2 + ADP + Pi
2. ACC-biotin-CO_2 + acetyl-CoA $\longrightarrow$ ACC-biotin + Malonyl-CoA

Acetyl-CoA carboxylase is subject to short-term and long-term regulation. Short-term regulation includes covalent modification and allosteric interactions. Acetyl-CoA carboxylase exists in active and inactive forms. The active form is polymeric while the inactive form is protomeric. The equilibrium between these two forms controls the rate of FA synthesis. Citrate, whose concentration increases when mitochondrial acetyl-CoA accumulates, promotes the active form of ACC (Vernon et al., 1991). The high citrate level then activates ACC thereby stimulating FA synthesis. Palmitoyl-CoA, the product

of FA synthesis, is an allosteric inhibitor. Palmitoyl Co-A inactivates ACC at high concentrations thereby inhibiting FA biosynthesis.

In addition to metabolite allosteric regulation, ACC is also subject to covalent modification. Acetyl-CoA carboxylase is inactive when phosphorylated and active when dephosphorylated. Phosphorylation is the result of the action of two different enzymes - cAMP-dependent protein kinase, which is activated by cAMP, and AMP-dependent protein kinase, which is cAMP independent. Epinephrine and norepinephrine result in the phosphorylation of ACC via increases in cAMP. Insulin activates ACC through dephosphorylation. In the periparturient period, the nutritional status of the cow dictates that insulin levels are low. Insulin resistance also occurs. The overall response to low insulin levels, along with insulin resistance as a result of high GH levels, is the inactivation of ACC with a subsequent reduction in lipogenesis. In sheep adipose tissue explants, addition of GH to the culture blocks the stimulatory effect of insulin on ACC activity (Vernon et al., 1991). Growth hormone administration has been shown to reduce adipose tissue ACC mRNA and protein abundance (Beswick and Kennelly, 1998) and activity (Lanna et al., 1995) in lactating dairy cows.

Acetyl-CoA carboxylase is also under long-term regulation, which controls the rate of ACC synthesis and degradation. In addition to its short-term regulation of ACC through dephosphorylation, insulin also exerts long-term regulation by promoting the synthesis of ACC (Voet and Voet, 1995).

1.4.4.2 fatty acid synthase

Fatty acid synthase is a multifunctional enzyme responsible for the synthesis of 16-carbon FA chains from malonyl-CoA and acetyl-CoA. The overall reaction is:

$$\text{Acetyl-CoA} + 7\ \text{Malonyl-CoA} + 14\ \text{NADPH} + 7\ H^{+} \longrightarrow$$

$$\text{Palmitate} + 7\ CO_2 + 14\ NADP^{+} + 8\ \text{CoA} + 6\ H_2O$$

Like ACC, FAS is regulated by the hormonal and nutritional status of the cow. Insulin stimulates FAS activity (Vernon et al., 1991). In early lactation, when insulin concentrations are depressed, FAS mRNA and protein activity are reduced (Vernon et al., 1991). Growth hormone treatment causes significant reductions in FAS mRNA and protein abundance (Beswick and Kennelly, 1998) and activity (Lanna et al., 1995) in lactating dairy cows. Growth hormone probably exerts its actions through effects on insulin although Vernon et al. (1991) found no effect of GH on FAS activity of sheep adipose tissue explants maintained in culture containing insulin. Those authors concluded that alterations in FA synthesis rates induced by insulin and GH are due primarily to changes in ACC activity.

1.4.4.3 lipoprotein lipase

Lipoprotein lipase is an extracellular enzyme. In adipose tissue and skeletal muscle, it is synthesized in parenchymal cells and then transported to the capillary endothelium. In the capillary, it hydrolyzes the TG of chylomicrons and VLDL to free FA and monoglycerides. The free FA are taken up by the tissues and converted back into TG. To be functional, LPL must first be activated by apoCII that is contained in the chylomicron or VLDL.

The activity of LPL in the dairy cow is influenced by physiological state and nutrition. Shirley et al. (1973) reported that adipose tissue LPL activity decreased by 33% from gestation to 14 d into lactation. Growth hormone and NEFA may both play a role in the regulation of LPL. High levels of GH in the peripartum period may inhibit

LPL abundance, and thus activity. Beswick and Kennelly (2000) reported that the administration of exogenous GH reduced the mRNA abundance of LPL by 85%. Liesman et al. (1995) measured LPL activity from the same cows and reported that GH reduced activity by 90%. Growth hormone is believed to exert its effects by inhibiting the action of insulin (Bauman and Vernon, 1993). High concentrations of plasma NEFA, commonly seen at parturition, are believed to inhibit LPL activity (Fielding and Fielding, 1991). The reduction in adipose tissue LPL activity at parturition and early lactation ensures that TG are directed to the mammary gland for milk fat synthesis.

1.5 Periparturient protein metabolism

Homeorhetic adaptation of the transition cow involves changes in protein metabolism. Animals in early lactation are commonly in negative nitrogen balance as mobilization of body protein reserves is necessary to support high rates of milk protein synthesis. The loss of body protein may be the result of alterations in protein turnover, either through increases in protein degradation or decreases in protein synthesis. In addition to this, there is a shift in protein synthesis from carcass tissues to the mammary gland. Baracos et al. (1991) demonstrated that absolute skeletal muscle and skin protein synthesis was reduced by 20 and 42%, respectively, in lactating goats compared to non-lactating goats. In the lactating goats, the weight of the hind-limb as a proportion of carcass weight was reduced, and the contribution of muscle to total mass of the hind-limb was also reduced compared to the non-lactating goats. This reduction in muscle mass was likely a result of increased degradation as fractional protein synthesis rates did not differ between the two groups of goats.

Mobilization of protein reserves is likely triggered by endocrinological changes in response to energy and protein status. Lobley (1992) proposed a model in which protein synthesis and degradation are regulated by IGF-1 and insulin. When concentrations of insulin are low and AA are limiting, a situation commonly observed in the close-up dry cow, protein degradation predominates over synthesis with the net result being protein catabolism. The AA released from muscle can be used for milk protein synthesis, oxidation, or gluconeogenesis. As feed intake increases, IGF-1 synthesis is stimulated leading to a substantial elevation in protein synthesis coupled with a smaller increase in protein degradation, the net result being protein anabolism.

An indirect assessment of protein degradation can be made from the concentration of its end products - 3-methylhistidine (3-MH) and creatinine. 3-methylhistidine is formed from methylation of histidine. It is found in various tissues including skin and gut, but the largest contributor is skeletal muscle. As a result of skeletal muscle protein breakdown 3-MH is released into the AA pool and, in cattle, is eliminated rapidly from the body in urine and milk (Blum et al., 1985). 3-methylhistidine is not metabolized by any body tissues upon its release from muscle and can therefore be used as an index of muscle breakdown. Blum et al. (1985) reported that plasma 3-MH concentrations began a rapid ascent 1 wk before calving and peaked 1 wk after calving. Concentrations then dropped dramatically to reach a low at 5 wk postpartum that was below the level 2 wk before calving. The peak around calving is probably the result of insufficient feed intake to meet the sudden increase in AA and glucose required for milk synthesis and gluconeogenesis. Concurrently, insulin levels are low, which would allow enhanced protein degradation. The peak in 3-MH following calving is probably also the result of

uterine involution, as smooth muscle also contains considerable amounts of 3-MH (Blum et al., 1985).

Urinary creatinine is an indicator of total muscle mass because its excretion is proportional to muscle mass. Urinary creatinine concentration declines from late gestation to early lactation (Simmons et al., 1994; VandeHaar et al., 1999) indicating reduced muscle mass as a result of increased mobilization of body protein reserves. The urinary 3-MH:creatinine ratio is used as a measure of myofibrillar protein degradation adjusted for muscle mass. In the study of Simmons et al. (1994), the 3-MH:creatinine ratio increased between d -7 and d 6 relative to parturition, suggesting that protein degradation increased after parturition. This finding is consistent with the lower urinary creatinine content. The postpartum loss of muscle mass, measured as urinary creatinine, and the increase in degradation, measured as the 3-MH:creatinine ratio, were greater in cows treated with GH during the prepartum period than in untreated cows (Simmons et al., 1994). This increase in postpartum protein degradation led the authors to suggest that the GH-treated cows had greater labile protein reserves during the prepartum period as a result of the GH treatment.

1.6 Nutritional needs of the dry cow

1.6.1 Nutrients required for conceptus growth

Gestational nutrient demands include those of the conceptus (fetus, placenta, and fetal fluids) and the uterus. The primary metabolic fuels of the fetus and associated tissues are glucose, lactate, and AA (Bell, 1995). Fatty acids and ketones are only minor contributors due to limited placental transport. Acetate's contribution as a fetal fuel is at most 10 to 15% (Comline and Silver, 1976). Glucose placental transfer occurs through

facilitated diffusion and is dependent on a concentration gradient for successful transfer. Conversion of glucose to lactate and fructose by the placenta may contribute to maintenance of the maternal-fetal gradient. Even when the dam is experiencing optimal nutrition, glucose only supplies 60% to 70% of fetal energy requirements (Reynolds et al., 1986). Apart from acetate, the remainder is supplied from AA. It is estimated that only one-third of the AA nitrogen taken up by the fetus is used for protein deposition (Bell, 1995) with the remaining two-thirds being oxidized. In late gestation, when the cow's DMI is reduced and the maternal glucose supply is limiting, the fetus relies heavily on AA as an energy source. The fetus will continue to extract AA from the maternal supply regardless of the dam's nutritional state because the AA are extracted by active transport. Unlike placental glucose transport, AA uptake is not dependent on a concentration gradient. Regardless of the fetus's capacity to extract maternal AA, the outcome of chronic maternal malnutrition is compromised fetal growth.

1.6.2 Nutrient recommendations for the dry cow

The NRC (1989) recognizes increased nutrient demand for gestation by allotting a one-step increase in requirements for the last two months of pregnancy. The increase in requirements specified by the NRC (1989) is substantial. For example, the maintenance net energy of lactation (NE_L) and CP requirements for a non-pregnant 650 kg cow are 10.3 Mcal/d and 428 g/d, respectively. For the same cow during the last two months of gestation, the requirements increase to 13.39 Mcal/d NE_L and 1281 g/d CP. This represents an increase in requirements of 30% for NE_L and 200% for CP. However, this approach does not coincide with fetal growth and mammary gland development, which are exponential. Using energy balance measurements, Moe and Tyrrell (1972) found that

the cow's energy requirement at 200 d, 250 d, and 280 d of gestation was 17%, 41%, and 74% higher, respectively, than that of a non-pregnant cow. These findings suggest that the NRC (1989) overestimates energy requirements in the far-off dry period, and increasingly underestimates requirements as calving approaches. The underestimation of the cow's energy requirements coupled with the decline in DMI has the potential to induce metabolic disorders during the periparturient period if cows are fed to the NRC (1989) standards.

The NRC (1989) may also underestimate protein needs of the cow during late gestation. The NRC requirement represents an average value for the dry period, and so as gestation progresses, and the needs of the conceptus increase, the cow may become increasingly protein deficient. The need for protein in late gestation is further increased by the growth and development of the mammary gland as well as prepartum colostrum synthesis. These increased demands for protein may result in the cow mobilizing labile protein reserves during gestation. If the assumption is made that at 275 d of pregnancy the protein intake of the cow is just sufficient to meet maintenance needs, then using the requirement equation of the Cornell Net Carbohydrate and Protein System (CNCPS, 2000) for pregnancy and the recommendation by Bell et al. (2000) for mammogenesis, and an assumed efficiency of 0.33 with which mobilized protein is used for pregnancy, then 485 g/d of body protein are required to meet all metabolic needs.

In response to knowledge acquired since the NRC (1989) regarding fetal growth and metabolic adaptations of the close-up dry cow, the NRC (2001) developed separate recommendations for the far-off dry and the close-up dry cow. For cows greater than 190 d pregnant, metabolizable protein (MP) requirements for pregnancy are calculated as a

function of days pregnant and expected calf weight (Bell et al., 1995). In agreement with NRC (1989), a 12% CP diet is still recommended, assuming that the requirements for RUP and RDP are met. This recommendation however does not take into account the protein requirements of the mammary gland. If mammary gland parenchymal mass increases by 460 g/d during the transition period (Capuco et al., 1997) and mammary parenchymal mass is 10% crude protein, an additional 120 g/d of MP would be required to support this growth. Assuming average DMI of 12 kg/d and a conversion of CP to MP of 70% (NRC, 2001), the CP density of the diet would have to increase to 13.4% during the last few weeks of gestation.

The NRC (2001) energy requirements for the close-up dry cow have been increased substantially from those recommended by NRC (1989). The recommended NE_L density of the close-up dry diet for mature cows is now 1.62 Mcal/kg, up from the 1.25 Mcal/kg previously recommended. This recommendation is based on the recognition of the decline in DMI of the precalving cow, energy requirements for mammary growth, potential beneficial effects on rumen VFA absorptive capacity, and a possible reduction in lipid mobilization and lipid-related metabolic disorders.

While the energy and protein requirements of the pregnant cow are substantially higher than that of the non-pregnant cow, they represent only a small proportion of those required by the lactating cow. Bell (1995) estimated that in a cow producing milk at a rate of 30 kg/d, the glucose and AA requirements of the mammary gland at 4 d postpartum were, respectively, 2.7 and 2.0 times those of the gravid uterus in late gestation. However, the cow's DMI does not increase in proportion to the increase in requirements. The estimated increase in glucose uptake by the lactating mammary gland

compared to that of the gravid uterus is approximately 1100 g/d while the increase in glucose supply is only approximately 600 g/d. Clearly there is a need for glucose precursors to eradicate this deficit. The dietary precursors most readily available are propionate and AA, but even if these are available exclusively for hepatic gluconeogenesis, a shortfall in glucose still remains. Bell (1995) estimated that the increase in supply of AA between early lactation and late gestation is equivalent to the difference in uptake between the lactating mammary gland and the gravid uterus, but that the total amount available was considerably less than the total required due to the need for gluconeogenesis. The major shortfall in the supply of glucose and AA during the first few weeks of lactation dictates that the cow must mobilize tissue reserves, both lipid and protein, during this period.

1.7 Body tissue mobilization

Because of the deficiencies in glucose and AA availability described above, cows in late gestation and early lactation mobilize substantial amounts of lipid and protein from their tissues. Postpartum protein mobilization contributes to milk protein synthesis as well as gluconeogenesis. Bell et al. (2000) estimated that cows producing 30 kg milk/d with a protein content of 4.2% mobilize 1000 g/d of body protein during the first 7 to 10 d of lactation, 500 g of which are needed for glucose synthesis. Assuming that the efficiency of conversion of body protein to milk protein is 80%, then 400 g/d of milk protein may be derived from the cow's labile protein reserves. This value agrees with the estimates of Wilson et al. (1988), who suggested that body protein may contribute as much as 34% of the casein and 24% of the lactose in milk in early lactation (35 DIM).

For the cow described above, this would be equivalent to 340 g/d of milk protein and 330 g/d of lactose.

Several estimates have been made of the amount of body lipid and protein available for mobilization. In the study of Komaragiri and Erdman (1997), cows producing 41 kg milk/d mobilized 21 kg of body protein and 53 kg of fat from 14 d prepartum to 35 d postpartum, regardless of dietary protein intake. Cows did not mobilize body protein beyond 5 wks, but continued to lose body fat. From 5 to 12 wk, cows on the 16% CP diet mobilized 14 kg of fat whereas the cows on the 19% CP diet mobilized 22 kg of fat. The greater fat loss of the cows on the 19% CP diet is probably a result of their higher milk yield (42.4 kg/d vs. 39.8 kg/d) and equivalent DMI (21.2 vs. 21.4 kg/d). Botts et al. (1979) measured protein reserves in lactating dairy cows (14 to 21 d postpartum; 24 kg/d milk during repletion stage) via a nitrogen depletion-repletion study. During the nitrogen depletion phase, they fed a 9% CP diet to deplete the animals' protein reserves, and during the repletion stage, they fed 14%, 18% or 22% CP diets. The cows were considered nitrogen-repleted when nitrogen balance approached equilibrium. In cows fed 18% or 22% CP diets during the repletion stage, body protein available for mobilization was 21 kg and 24 kg respectively, equivalent to 25 and 27% of estimated total body protein. Although labile protein stores may be of this magnitude, it is unlikely that the periparturient cow would ever deplete her entire protein reserve, short of starvation. Tamminga et al. (1997) reported body protein mobilization of only 4.6 kg during the first four wk of lactation and replenishment of 2.2 kg of body protein reserves from 4 to 8 wk postpartum in cows producing 35 kg of milk daily. This data must be viewed with some caution, as there were no direct measurements of body composition. Estimations of body

fat and protein were based on the calculated energy balance and assumed energy contents of each of the tissues, thus these estimates of mobilization may be erroneous. Andrew et al. (1995), using serial slaughter techniques, found no difference in body protein content between 7 d before calving and 63 d after calving. This finding does not preclude body protein loss, as it is entirely possible that protein reserves were mobilized in the first few weeks of lactation and replenished by 63 days in milk (DIM). Body fat loss in this study was 42.4 kg, greater than the 30.9 kg reported by Tamminga et al. (1997) but less than that reported by Komaragiri and Erdman (1997). Differences in milk yield among the studies probably explain the differences in fat mobilization.

The body composition studies (Komaragiri and Erdman, 1997; Andrew et al., 1995) demonstrate that as cows progress in lactation the composition of mobilized body tissue changes. The proportion of fat to protein increases and lipid stores continue to be depleted while protein stores are being repleted. Thus, as lactation continues, the energy content of mobilized tissue increases.

The amount of energy that the cow mobilizes is substantial. In the study of Komaragiri and Erdman (1997), cows mobilized 605 Mcal of body energy by 5 wk postpartum. Eighty-one percent of the energy was derived from body fat and 19% from body protein. Andrew et al. (1995) reported a loss of body energy of 372 Mcal between 7 d prepartum and 63 d postpartum, and over the first 8 wk of lactation, Tamminga et al. (1997) reported mobilization of 307 Mcal of energy. Assuming that mobilized energy is utilized with an efficiency of 80%, and that one kg of 4% fat-corrected milk requires 0.74 Mcal of NE_L (NRC, 1989), then ~350 kg of milk can be produced from the cow's tissue reserves during the first 8 wk of lactation.

1.8 Effect of prepartum nutrient intake on the periparturient cow

A successful transition from the gestating to the lactating state is largely dependent on the cow maximizing energy and protein intake throughout the periparturient period so that mobilization of tissue reserves prepartum and the occurrence of metabolic disorders are minimized.

1.8.1 Energy intake

In the far-off dry period, cows are normally fed high-forage, low-energy diets, but as parturition approaches, lead-feeding, or the introduction of concentrates into the diet, is commonly practiced. This management practice offers several benefits. First, the incorporation of grain in the diet, which is a source of rapidly fermentable carbohydrate, will increase the energy content of the diet and help to maintain the cow's energy intake during the prepartum decline in feed intake. Secondly, feeding diets high in fermentable carbohydrate helps adapt the rumen microflora to high-concentrate postpartum diets, promoting ruminal papillae development and increasing the absorptive capacity of the rumen epithelium (Dirksen et al., 1985; Goff and Horst, 1997). These changes allow improved absorption of volatile fatty acids and help prevent a decline in rumen pH, thereby reducing the risk of cows developing rumen acidosis and reducing the possibility of cyclic feed intakes in early lactation.

Energy status of the peripartum cow appears to be improved with increased energy intake prepartum. Most studies in which diets were fed that had energy densities higher than that recommended by NRC (1989) have shown reductions in NEFA (Dann et al., 1999; Minor et al., 1998; VandeHaar et al., 1999), liver TG content (Minor et al., 1998; VandeHaar et al., 1999) and BHBA concentrations (Minor et al., 1998) around calving.

Increased plasma IGF-1 concentrations have also been reported (VandeHaar et al., 1999). In cows that were fed diets with high levels of non-fibre carbohydrate (NFC), both before and after calving, NEFA, BHBA, and liver TG at d 28 were reduced, and liver glycogen levels increased, compared to those of cows fed diets with more fibrous carbohydrates (Minor et al., 1998). In most instances, improved energy status of the cow prepartum has not translated into improved production (Dann et al., 1999; VandeHaar et al., 1999; Dewhurst et al., 2000).

An alternative to feeding more grain prepartum to increase energy intake is to process the grain so that it is more ruminally available. In the study of Dann et al. (1999) this was achieved by steam-flaking corn as opposed to feeding it cracked. Feeding steam-flaked corn for 28 d prepartum reduced blood urea nitrogen and NEFA concentrations prepartum, but did not affect milk or milk component yields. The reduction in NEFA concentration indicates a reduction in body fat mobilization and more efficient utilization of dietary energy.

Grummer (1995) suggested that cows that are in less negative energy balance before calving would be in better energy balance after calving, however, VandeHaar et al. (1999) did not find such a relationship. In their study, cows on a high energy diet before calving were in better energy balance prepartum than cows on a low energy diet but postpartum there was no difference in energy balance between the two groups. Minor et al. (1998) reported similar findings.

Energy intake prepartum affects protein metabolism in addition to energy metabolism. Increasing the energy and protein content of the diet from 1.30 Mcal/kg NE_L and 12.2% CP to 1.61 Mcal/kg NE_L and 16.2% CP increased the urinary 3-

MH:creatinine ratio during the last 2 wk prepartum (VandeHaar et al., 1999). The authors suggested that the more energy-dense diets supported greater protein synthesis since increased muscle degradation, as evidenced by the higher 3-MH:creatinine ratio, is often accompanied by increased muscle synthesis. Increasing the protein content of the diet without increasing the energy content had no effect on the 3-MH:creatinine ratio.

1.8.2 Protein intake

Increasing the CP and/or RUP content of prepartum diets generally has no effect on prepartum DMI (Van Saun et al., 1993; Huyler et al., 1999; Greenfield et al., 2000). In these studies, the pattern of prepartum feed intake varied. In the studies of Van Saun et al. (1993) and Greenfield et al. (2000), the cows experienced a gradual decline in intake one to two weeks prior to calving, whereas the cows in the study of Huyler et al. (1999) had no depression in prepartum intake.

Increasing total CP content or RUP content of the prepartum diet has not produced consistent results. Hook et al. (1989) reported a milk yield increase in heifers fed a 13% CP diet compared to a 9% CP diet for 60 d before calving. Van Saun et al. (1993) conducted a study with first calf heifers in which RUP content of the prepartum diet was increased from 27% to 39% (and CP from 12.4% to 15.3%). Increasing the amount of RUP in the prepartum diet did not increase milk yield but increased milk protein content by 0.22 percentage units. Heifers on the high RUP diet maintained their body condition until calving while those on the low RUP diet lost condition. The authors speculated that the heifers fed additional RUP relied less on mobilization of labile protein pools to meet fetal and maternal growth requirements in late gestation. It is possible that mature cows would not have responded in the same way because they no longer have a requirement for

growth. There was no effect on BCS of multiparous cows fed one of three levels of CP (10.6% to 14.5%) prepartum (Putnam and Varga, 1998). Huyler et al. (1999) fed three levels of RUP (11.7 and 3.1%, 15.6 and 6.8%, and 20.6 and 10.6% CP and RUP, respectively) to prepartum multiparous cows and found no differences in DMI or BCS throughout the periparturient period. Cows receiving the lowest RUP diet tended to produce 3 kg/d less milk than the cows on the other two treatments. The prepartum treatments had no effect on plasma NEFA or liver TG. Nonesterified fatty acids were elevated at calving even though there was no feed intake depression before calving, suggesting that the increase was hormonally induced.

In contrast to the studies of Van Saun et al. (1993) and Huyler et al. (1999), Wu et al. (1997) increased RUP content of the prepartum diet while leaving CP constant (14%). Dry matter intake as a percentage of BW was higher for the cows receiving the high RUP diet but milk yield was unaffected by treatment. Putnam and Varga (1998) increased the CP content of the prepartum diets but kept the ratio of RDP to RUP constant. In agreement with the previously mentioned studies, milk yield was not improved by the dietary treatments. As in the studies of Huyler et al. (1999) and Greenfield et al. (2000), NEFA concentrations were unaffected by diet. This was surprising because glucose concentrations increased as CP content of the diet increased. With higher glucose concentrations, indicative of increased carbohydrate digestion, the cow's reliance on body fat mobilization decreases and NEFA levels would be expected to be lower.

Milk (40.8 kg/d vs. 38.0 kg/d) and milk fat and protein yield during the first 35 DIM were higher for cows receiving a 12% CP diet (26% RUP as % of CP) prepartum than for cows receiving a 16% CP diet (33% or 40% RUP) (Greenfield et al., 2000). This

may have been the result of the cows on the 12% CP diet eating more in the immediate postpartum period. The authors suggested that the lower intake with the high protein diets was the result of prolonged hepatic AA catabolism. Hartwell et al. (2000) reported a similar result. In their study cows were fed prepartum diets containing 4% RUP as % of DM (14% CP) or 6.2% RUP (16.2% CP) with RDP constant (10% of DM). Milk yield during the first 56 DIM was higher for the cows consuming the low RUP diet. The cows on the low RUP diet also had higher postpartum DMI and lost less BW during the first 8 wk of lactation. Both Greenfield et al. (2000) and Putnam and Varga (1998) suggested that NRC (1989) recommendations for protein content of the dry cow diet (12%) were adequate. While NRC (2001), which recognizes the decline in DMI prepartum and the increased conceptus demands, still recommends that the close-up dry diet for multiparous cows contain only 12% CP, it does make note that higher protein density may be beneficial for the last 3 to 5 d before calving.

Efforts to improve lactational performance by increasing CP or manipulating RUP in the prefresh period are hampered by the fact that the AA requirements of the transition cow are unknown. As stated by the NRC (2001) "strategic supplementation of limiting amino acids may prove to be more successful than increasing total CP or ruminally undegradable protein". Studies have been conducted where AA have been supplemented postruminally in the transition period. Bach et al. (2000) fed 60 g/d of ruminally protected Met to prefresh cows and found that arterial urea concentrations decreased, and Met supply to the peripheral tissues increased due to a shift from splanchnic uptake to splanchnic release. These findings led the authors to suggest that Met was the limiting AA in the control diet, and that Met supplementation during the prepartum period may be

beneficial. The authors did not report on lactational performance postcalving. In the study of Oldham et al. (1984), 300 g/d of casein infused abomasally starting at 9 d postpartum increased milk yield by 2.2 l/d and milk protein yield by 98 g/d. Chew et al. (1984) infused arginine (Arg) intravenously for 7 d precalving and reported that milk yields tended to be higher for the first 22 wk of lactation. These studies indicate that the AA supply of the basal diets was not optimal for animal performance, and that productivity could be improved by supplying additional AA.

Although the transition period sets the stage for the cow's performance in the subsequent lactation, considerably more effort has been put into determining the AA needs of the lactating cow. This is not surprising as the demand for AA is much higher during lactation than during the dry period, and improved profits can be realized through efficient feeding of the lactating cow. Therefore, the balance of this review will focus on AA metabolism of the lactating cow and of other species where there is a paucity of information on the dairy cow.

1.9 Methods to determine amino acid requirements

Proteins consist of twenty primary AA, half of which are considered essential. Essential AA (EAA) either cannot be synthesized by the animal, or are synthesized at rates insufficient to meet requirements. The AA considered essential for the dairy cow include Arg, His, isoleucine (Ile), leucine (Leu), lysine (Lys), Met, phenylalanine (Phe), threonine (Thr), tryptophan (Trp) and valine (Val). The nonessential AA (NEAA) can be synthesized by the animal from other metabolic intermediates and, as such, are not generally considered to be limiting for protein synthesis. This was demonstrated in the study of Metcalf et al. (1996), where an intravenous infusion of EAA (in the same

proportions as found in milk protein) was equally effective at increasing milk protein yield as an infusion of EAA plus NEAA. However, supplementation of NEAA may have a sparing effect on EAA. Arginine, an EAA, is taken up by the mammary gland in amounts greatly exceeding its secretion in milk (Bickerstaffe et al., 1974; Mackle et al., 2000). One of the reasons for this high extraction is synthesis of proline, a NEAA (Mepham, 1982). Proline infused into the duodenum of early and mid lactation cows increased milk fat content and yield for all cows and improved milk protein yield in mid lactation cows (Bruckental et al., 1991). It also reduced Arg uptake by the mammary gland by 40 to 50%.

Over the years, several production and physiological parameters have been used as indices of AA limitation.

1.9.1 Nitrogen balance and milk production response

Measurement of animal production, either milk yield, milk protein yield, or tissue gain, in response to graded levels of AA supplementation is probably the simplest method of determining the degree and sequence of AA limitation. This method has been used widely in dairy cattle. A basal diet deficient in protein is fed, and an individual AA or combination of AA is infused intravenously or abomasally. The milk yield and milk component yield response is then measured. Schwab et al. (1976) used this method in a series of experiments to determine that Lys and Met are the first limiting AA when corn-based diets are fed. In addition, dietary nitrogen intake can be compared to nitrogen outflow in feces, urine, and milk. When intake equals output, the animal is in nitrogen equilibrium. Upon incremental addition of the first limiting AA, nitrogen balance increases until a plateau is reached. The drawback to this method is that the response will

vary with factors such as the energy and protein content of the basal diet, the balance of AA in the diet (Schwab et al., 1976), and the stage of lactation (Istasse et al., 1986). As stated by Schwab et al. (1976) "because of the differences in feed proteins with respect to amino acid composition and the extent of their degradation in the rumen, ingredient composition of the ration will influence which amino acids are most limiting for milk production and/or milk protein synthesis".

Another difficulty with this method is trying to accurately establish the point of maximum response. For example, if milk protein yield is used as the response, with each increment of AA intake the response declines (Harper, 1994). For example, when casein was infused in incremental amounts from 0 to 600 g/d, the initial 200 g increment resulted in an increase in milk protein of 81 g/d, while the last 200 g increment only resulted in a 31 g/d increase in milk protein (Whitelaw et al., 1986). However, when AA are supplemented, other anabolic responses, such as nitrogen retention, should be used in conjunction with milk production as the measured response. In the casein study cited above, the first 200 g increment improved nitrogen retention by 6 g/d (protein retention of 38 g/d) while the last 200 g increment improved nitrogen retention by 7.8 g/d (protein equivalent of 49 g/d). Therefore, the change in milk protein in conjunction with the change in nitrogen retention gives a better indication of the total efficiency of AA use.

1.9.2 Plasma amino acids

Plasma AA concentrations have been used as indicators of AA limitation because the AA concentration will respond incrementally as the AA supply changes from inadequate to adequate (Harper, 1994). The response is referred to as a 2-phase broken line response (Figure 1-1). However, as the AA supply increases above its requirement,

the change in plasma concentration does not parallel the change in supply because of homeostatic processes that regulate the plasma concentrations (Ball, 1996). For example, the liver plays a central role in determining the release of AA into the peripheral circulation (Lobley and Milano, 1997). As dietary AA supply increases, the liver will extract a greater percentage of AA presented to it, such that arterial concentrations may not increase (Guerino et al., 1991).

Another problem with the use of plasma AA concentrations is that the plasma AA pool represents only a small proportion of total AA in the body, and plasma concentrations do not necessarily reflect tissue concentrations. Plasma concentrations are a reflection of protein synthesis and degradation, splanchnic and peripheral tissue uptake and release, and AA catabolism. In fact, an increase in plasma concentration may not reflect excess of the AA at all. For example, during starvation, branched-chain AA (BCAA) are released from muscle resulting in higher plasma concentrations (Bergen, 1979). Conversely, low concentrations, which may be interpreted as a dietary deficit, may also indicate increased uptake into protein.

When limiting AA are infused postruminally into lactating dairy cows, it would be expected that plasma concentration of the other AA would decline as a result of increased milk protein synthesis. However, Schwab et al. (1976) found no, or only minimal, changes in AA plasma concentrations when limiting AA were infused even though milk protein secretion did increase. Their data suggest that the use of plasma AA concentrations as indicators of AA limitation be used with caution.

1.9.3 Plasma urea

The theory behind the use of plasma urea concentrations is that when one AA is limiting, the other AA are in excess and will be catabolized yielding urea (Ball, 1996). As the supply of the first limiting AA meets requirements, the concentration of urea will decline. Limitations of this method include underestimation of the uptake of dietary AA for protein synthesis due to catabolism of endogenous AA, difficulty in detecting small metabolic changes because the plasma urea pool is so large, and variation in urea cycling (Ball, 1996). Additionally, in ruminants, urea production from AA catabolism is diluted with ammonia detoxification and will make the use of plasma urea concentrations even less sensitive.

1.9.4 Amino acid oxidation

Both direct oxidation and indicator oxidation of AA have been used as a means of determining an animal's AA requirements. In the direct oxidation method, the suspected limiting AA is provided in graded levels (by increasing its dietary concentration) and its oxidation is measured by giving a ^{13}C or ^{14}C-labeled AA and monitoring the labeling of CO_2 to determine the AA oxidation. The theory behind this method is that the extent of oxidation increases as the AA supply changes from deficient to excess. When the AA is deficient, it is used efficiently for protein synthesis and the oxidation rate will be minimal. As the AA supply exceeds requirement, oxidation will increase. The point at which oxidation begins to increase indicates the requirement. However, as summarized by Ball (1996), there are several drawbacks to this method. As the supply of the AA is increased, the pool size of the AA increases resulting in a dilution of the labeled AA. Secondly, if the AA is inappropriately labeled, the label may be distributed to metabolites

other than CO_2. The label must be on a carbon atom that will be lost as CO_2 when the AA is catabolized.

The indicator oxidation method offers advantages over the direct oxidation method in that recovery conditions need to be optimized for only one specific AA, and the requirements for AA with complex metabolism can be determined. With this method, the oxidation of an indicator AA is used to estimate the requirement for a different AA. When an AA is limiting, all of the other AA are supplied in relative excess and thus will be oxidized. As the supply of the limiting AA is increased, protein synthesis will increase and oxidation of the other AA, including the indicator AA, will decrease until the requirement for the AA is met. When the AA is provided in amounts beyond its requirement, there will be no further decrease in oxidation of the indicator AA.

1.9.5 Amino acid extraction and transfer efficiency

Derrig et al. (1974) attempted to identify limiting AA by measuring their uptake by the mammary gland and calculating the expected milk protein yield if the uptake of individual AA was used exclusively for milk protein synthesis (they used the uptake:output ratio as an indicator of AA deficiency). Amino acids with a ratio of unity would be considered most limiting. Using this method, they determined that Phe was most limiting, followed by Met. However, this method is not valid because of differential mammary gland AA metabolism. For AA whose uptake equals milk output, an increase in milk protein synthesis must be accompanied by an increase in mammary gland uptake. This does not indicate, however, that these AA are limiting because the mammary gland has the potential to increase its extraction rate of AA. Amino acids that are taken up in excess of their milk output are catabolized in the mammary gland. If these AA were

limiting for milk production, they probably would not be catabolized. However, if catabolism is the priority over milk protein secretion, then these AA could become limiting for protein synthesis. The uptake:output ratio would give no indication of this and is therefore unsuitable as a method of predicting AA deficiencies. For example, lysine is often considered a limiting AA for milk protein synthesis when corn-based diets are fed, but the mammary gland uptake:output ratio is greater than one (Guinard and Rulquin, 1995), which would suggest that the AA is not limiting.

1.10 Current systems for predicting protein requirements of dairy cattle

For many years the protein requirement of dairy cows was expressed as CP. However, milk yield and milk protein content are poorly predicted from the CP content of diets in the range normally fed to lactating cows. The NRC (2001) found that the correlation between milk yield and CP content of the diet (in conjunction with DMI) was only 0.29, and there was no correlation between milk protein content and dietary CP content. In 1989, the NRC expressed protein requirements as absorbed protein (AP), which was the digestible true protein arising from ruminally synthesized microbial crude protein plus RUP. The AP requirement included the protein needed for maintenance, fecal metabolic nitrogen, and production (NRC, 1989). The AP system required that the rumen degradability of the feedstuffs be known, so that the contribution of microbial protein and RUP to AP could be calculated. At the time that the NRC (1989) was published there was only limited data on the degradability of different feedstuffs, so use of the AP system was limited. The AP system also suffered from other drawbacks, primarily that a fixed intestinal digestibility was assigned to RUP and that endogenous CP was not considered to contribute to AP, both of which would lead to errors in

predicting AA supply. The greatest limitation to the system was that the AA composition of the AP was not taken into account. Considering that the cow's protein requirement at the tissue level is indeed an AA requirement, any system that bases requirements on protein will be inadequate. The latest edition of the *Nutrient Requirements of Dairy Cattle* (NRC, 2001) addresses the need for expressing the animal's requirements as AA, but because of a paucity of data, the requirements are still expressed in terms of protein, specifically metabolizable protein. Metabolizable protein is defined as "the true protein that is digested postruminally and the component AA absorbed by the intestine" (NRC, 2001). It is equivalent to AP but the use of the MP term avoids the implication that proteins are absorbed. The NRC (2001) differs from the previous edition in terms of predicting protein supply to the cow. Microbial CP flow is predicted from digestible organic matter (OM) intake, not net energy intake. Regression equations have been included for predicting the degradability of feedstuffs and include factors such as DMI, forage:concentrate ratio in the diet, and neutral detergent fibre (NDF) content of the diet. Intestinal digestibility of RUP is no longer fixed, but varies with the feedstuff in question. Endogenous protein is now considered to contribute to MP supply. The biggest improvement comes in the area of AA supply. The NRC (2001) incorporates regression equations that predict the duodenal flow of EAA based on the EAA profile and rumen degradability of individual feedstuffs. The contribution of individual AA to MP is also calculated.

Although the NRC (2001) is an improvement over the previous edition, it still makes no attempt to predict AA requirements, inevitably because of the paucity of research in this area. The majority of the research on AA requirements has focused on

Met and Lys, which are often referred to as being first and second limiting. As a result, Met and Lys requirements are given, expressed as percentages required in MP. If the MP requirement is exactly met, this approach has merit. If, however, MP is deficient or grossly excessive, then this method is of limited value, and requirements would be better expressed as grams/day.

1.11 Splanchnic modulation of amino acid supply

While the MP system is an improvement over the CP system, it is unable to predict the availability of AA for milk protein synthesis. Metabolizable protein is an indicator of protein disappearance from the gut, not an indicator of the amount of protein that is available to the peripheral tissues. Large metabolic transformations of absorbed nutrients take place in the portal-drained viscera (PDV) and liver. Amino acids are used by the gut epithelium during absorption, either as energy substrates or for protein synthesis (Seal and Reynolds, 1993), and consequently, disappearance of AA from the gut is not equivalent to their appearance in the portal vein. Tagari and Bergman (1978) reported that 30% to 80% of AA disappearing from the small intestine did not appear in the portal vein. Berthiaume et al. (2001) reported that in lactating dairy cows, PDV flux of EAA was only 61% of their intestinal disappearance, and PDV flux of the NEAA was only 38% of intestinal disappearance. Similarly, MacRae et al. (1997), using lambs, found that PDV flux of EAA was 69% of their disappearance from the small intestine. In both studies intestinal disappearance of EAA was equivalent to mesenteric-drained visceral (MDV) flux. This does not suggest that AA were not catabolized by the small intestine, but that the quantity of AA arising from endogenous secretions is equivalent to the amount of AA catabolized by the intestinal tissues. Portal drained visceral fluxes of the

EAA averaged 62% of the MDV fluxes in both dairy cows (Berthiaume et al., 2001) and sheep (MacRae et al., 1997). A large proportion of this apparent loss is the important contribution of endogenous secretions to the duodenal flux. These secretions originate from PDV, non-MDV tissues but are absorbed into the mesenteric vein. Endogenous secretions represent 10% to 20% of duodenal nitrogen flow (Demers et al., 1999), thereby supplying an additional quantity of AA to the mesenteric vein. In addition, some oxidation of AA occurs contributing to the difference between PDV and MDV flux.

While MDV flux of EAA is equal to intestinal disappearance, the same does not hold true for the NEAA. Berthiaume et al. (2001) reported that intestinal disappearance of NEAA was 131% of MDV flux. Aspartate and glutamate (Glu) disappeared from the small intestine in the greatest amounts, but relative to their intestinal disappearance, only very small amounts appeared in the mesenteric vein, indicating their importance as metabolic fuels for the gut (Reynolds et al., 1994).

Reynolds et al. (1994) suggested that the degree to which AA are limiting for milk protein production is partly determined by their metabolism in the PDV. While this certainly is true, the degree of limitation is further controlled by hepatic extraction and release of AA. For example, Met is commonly referred to as being the first limiting AA for milk protein synthesis (Schwab et al., 1976). Not only is PDV flux of this AA less than 70% of its intestinal disappearance, but hepatic extraction is high, averaging 31% of PDV absorption in lactating cows (Lapierre et al., unpublished). Conversely, the BCAA – Leu, Ile, and Val – which are seldom considered limiting for milk protein synthesis, are either released by the liver (Lapierre et al., unpublished) or are extracted in very small

amounts. As a result, the amount available for use by the peripheral tissues nearly mimics the amount released from the PDV.

Glutamine (Gln) and Glu are unique among the AA in terms of their metabolism in the PDV and liver (the splanchnic tissues). Portal-drained visceral flux of Gln is often negative (Huntington and Prior, 1985; Reynolds and Huntington, 1988; Reynolds et al., 1992; Seal and Parker, 1996; Berthiaume et al., 2001), although net release has been reported (Reynolds and Huntington, 1988; Reynolds et al., 1988; Balcells et al., 1995). In the gut, Gln is used for both energy and alanine production. Release from kidneys and peripheral tissues such as muscle is the source of the Gln. The liver also extracts Gln (Reynolds et al., 1994). Glutamate, which is present in high amounts in ruminant diets, is either removed by the gut (Bergman, 1986) or absorbed in small quantities (Berthiaume et al., 2001), and is released by the liver. Thus, the shuttle of Glu and Gln between the liver and peripheral tissues allows for transport of ammonia in a non-toxic form to the liver where it is converted to urea.

Alanine is released by the PDV in very high amounts (Huntington et al., 1988; Berthiaume et al., 2001) but substantial amounts are removed by the liver (Lapierre et al., unpublished) and kidney and are used for gluconeogenesis. In sheep, liver removal of alanine exceeds PDV release, with the balance arising primarily from release from muscle (Bergman, 1986). Alanine release from the peripheral tissues is a means to transport ammonia to the liver for detoxification and provide carbon for glucose synthesis.

It is evident that the liver extracts or releases individual AA to varying degrees. In general terms, NEAA are extracted by the liver, relative to PDV absorption, to a greater

degree than EAA. For example, Lapierre et al. (unpublished) reported that NEAA hepatic extraction relative to portal absorption was 44%, whereas EAA hepatic extraction was only 8%. The low extraction rate of the EAA is due primarily to release of the BCAA.

The release and removal of AA by the splanchnic tissues is dependent on the type and amount of diet fed. In sheep fed near maintenance, PDV release and liver extraction of AA were nearly equivalent (Bergman, 1986). As noted by Reynolds (1992), at maintenance intake, the proportion of absorbed AA used by the liver may be greater than at production intakes. In lactating dairy cows, whose energy and protein intake is considerably above maintenance levels, splanchnic release of the total sum of AA is positive. In late lactation (210 DIM), liver extraction was approximately 27% of PDV release (Lapierre et al., unpublished), while in another study with cows in early lactation (76 DIM), liver extraction was 13% (cows on control diet; Lapierre et al., unpublished). In growing beef steers, high feed intake increased PDV release and liver extraction of α-amino nitrogen compared to low feed intake, but the increase in liver extraction was less than the increase in PDV release such that total splanchnic flux was higher with the high intake (Reynolds et al., 1992b). However, additional AA intake is not always associated with higher splanchnic release of AA. Guerino et al. (1991) infused steers with casein and found no improvement in splanchnic release of α-amino nitrogen compared to steers that were not infused. Similarly, Bruckental et al. (1997) reported no increase in splanchnic flux of AA when they infused steers with casein. There are two possible explanations for these results. First, the possibility exists that the liver increased its protein synthesis when the additional AA were supplied. This protein synthesis must be

accompanied by an increase in retention (i.e., tissue growth) to account for the increased AA removal. If protein synthesis is increased without a concomitant increase in nitrogen retention, then protein degradation must also have increased, resulting in no effect on net flux. Alternatively, it may be that the liver simply acts as a sink to remove excess AA. The second explanation seems more likely because when GH was infused with the casein, splanchnic uptake of AA was reduced significantly from 304 mmol/h with the casein infusion to 68 mmol/h with the casein and GH infusion (Bruckental et al., 1997). This reduction in hepatic uptake resulted in an 80% increase in splanchnic flux. Because the GH-casein infusion increased nitrogen retention and hence the need for AA relative to that of the casein infusion, the possibility exists that GH exerted regulatory effects on hepatic uptake of AA and signaled the liver to reduce its extraction of AA. Alternatively, the GH signal may have been directed to the peripheral tissues, which increased their AA uptake thereby reducing AA removal by the liver.

Among studies, PDV release of α-amino nitrogen does not appear to be correlated with dietary nitrogen intake. Seal and Reynolds (1993) summarized data from nine experiments and found that the correlation between PDV α-amino nitrogen flux and nitrogen intake was very low ($r = 0.24$). This may be attributable to differences in energy intake and the resulting effect on microbial protein synthesis and duodenal flow. Similarly, it may be due to differences in the proportion of RDP and RUP in the diet. Increased RDP intake may have an effect on total AA splanchnic flux through changes in AA duodenal flow and possibly through changes in PDV ammonia absorption. In heifers fed casein (high RDP) vs. those fed corn gluten meal or blood meal (low RDP), there was a tendency for α-amino nitrogen PDV flux to be lower and ammonia PDV flux to be

higher (Huntington, 1987). When diets contain high levels of RDP, the proportion of nitrogen absorbed as ammonia increases while the proportion absorbed as AA decreases, thus reducing AA flux through the PDV and liver. The increased ammonia absorption from the PDV will also result in greater urea production. This is turn may reduce α-amino nitrogen flux through the splanchnic tissues because of the requirement for glutamate and aspartate synthesis, as well as for oxaloacetate synthesis, which is required for ureagenesis as well as gluconeogenesis (Reynolds, 1992). In sheep, supplementation of the diet with urea increased the conversion of alanine to CO_2 and glucose in isolated hepatocytes (Mutsvangwa et al., 1996). In contrast, Milano et al. (2000) found no effect of NH_4HCO_3 infusions in the mesenteric vein of sheep on AA flux through the PDV and liver.

In contrast to the effects of nitrogen intake, PDV release of α-amino nitrogen does appear to vary with the energy intake of the animal. Reynolds et al. (1992a) fed two levels of CP (11% and 16%) at two energy intakes to beef steers and found that PDV absorption was not influenced by protein intake, but was elevated with the high energy intakes. The effect of energy intake on PDV release of AA appears to vary with the source of the energy substrate. Intraruminal propionate infusion at 1.0 mol/d in steers increased net absorption of EAA and NEAA across the MDV, and net absorption of EAA across the PDV compared to animals infused with water (Seal and Parker, 1996). In contrast, in steers given butyrate infusions, there was either no change (Reynolds et al., 1992c), or a tendency ($P = 0.14$; Krehbiel et al., 1992) for PDV flux of α-amino nitrogen to decline with increasing butyrate. However, total splanchnic flux was unaffected because liver extraction decreased as the amount of butyrate infused increased. It appears

then that propionate reduces the gut's utilization of AA while butyrate increases utilization in the gut and/or peripheral tissues. Krehbiel et al. (1992) suggested that the additional butyrate increased butyrate oxidation for energy and increased AA metabolism for protein synthesis, although it was not reported if nitrogen retention changed as a result of the butyrate infusion. Overall, it appears that the effects of dietary protein and energy intake on splanchnic flux of AA are variable, and that more research is required before predictions of postsplanchnic AA availability can be made with confidence.

1.12 Amino acid metabolism in the mammary gland

1.12.1 Protein requirements for lactation

During lactation, the protein needs of the mammary gland far outweigh the protein needs for maintenance of other body tissues. For example, for a 600 kg mature cow producing 40 kg of milk containing 3.5% fat and 3.0% true protein, 68% of MP is required for lactation, while only 32% is needed for maintenance (calculated from NRC, 2001). Metabolizable protein for lactation is assumed to be used with an efficiency of 67% (NRC, 2001), thus the MP requirement for lactation is calculated as milk protein yield / 0.67. Exact AA requirements for lactation remain unknown. Several models predict AA requirements, the most well known system being the Cornell Net Carbohydrate and Protein System (O'Connor et al., 1993). This system calculates AA requirements from nitrogen requirements, AA composition of milk and tissue, and efficiency of AA use. Net protein required is the sum of protein needed for growth (protein in tissue), gestation (protein in conceptus), lactation (protein in milk), and maintenance (protein in scurf, urine, and feces). The AA requirements are calculated by multiplying the protein requirements by the AA composition of each of the products afore

mentioned, and dividing by the efficiency of use of each AA for each product. Efficiency values for maintenance were obtained from Evans and Patterson (1985) who assigned the values based on information from Oldham (1984), Harper (1983), and Lindsay (1982). Pregnancy and lactation efficiency values were also originally obtained from Evans and Patterson (1985) but have been modified for version 4 of the model (CNCPS, 2000). The lactation efficiency values are now based on mammary gland uptake to output ratios of the AA. Efficiency values for AA use are in Table 1-1.

1.12.2 Amino acid uptake by the mammary gland

The mammary gland synthesizes the majority of the proteins it secretes from free AA taken up by the gland. These proteins include casein and the whey proteins, β-lactoglobulin and α-lactalbumin. Other proteins, namely serum albumin and immunoglobulins, are synthesized outside the gland and are extracted intact (DePeters and Cant, 1992).

Studies by Bickerstaffe et al. (1974) and Mackle et al. (2000) show that the uptake to output ratio of total AA carbon and nitrogen by the mammary gland is reasonably balanced. However, differences exist between the EAA and the NEAA. The total sum of EAA are extracted in excess of their output in milk while uptake of NEAA is insufficient to account for their output in milk. Within the EAA, however, there is considerable disparity. Arginine and the BCAA are extracted in amounts far exceeding their output in milk (Bickerstaffe et al., 1974; Mackle et al., 2000), suggesting that they are metabolized within the gland for purposes other than direct utilization for protein synthesis. The excess uptake of Arg relative to its output in milk is evident immediately after parturition, which is unique among the EAA (Mepham, 1982), and suggests that the products of its

catabolism serve vital roles in the mammary gland. Several roles have been elucidated for Arg. It is a precursor for proline, whose uptake by the mammary gland is less than its output in milk (Mackle et al., 2000). It is also hydrolyzed to ornithine. Ornithine is an important AA within the mammary gland in that it supplies carbon for proline synthesis and nitrogen for several NEAA (Mepham, 1982). The BCAA are extensively catabolized within the mammary gland to yield organic acids, carbon skeletons for NEAA synthesis, and CO_2 (Bequette et al., 1998).

The uptake of His, Met, Phe, and Thr closely matches their output in milk suggesting that they are incorporated into milk protein almost exclusively (Mackle et al., 2000). Lysine is extracted in amounts exceeding its appearance in milk protein (Mackle et al., 2000; Guinard et al., 1995). This is an unusual situation for an AA that is considered limiting for milk protein synthesis, and suggests that Lys has other obligatory roles within the mammary gland, such as oxidation to yield ketogenic intermediates (Bequette et al., 1998). Because the uptake by the mammary gland of NEAA is less than their output in milk, the NEAA are obviously being synthesized within the gland. The precursors for their synthesis include the EAA that are taken up in excess, primarily Arg, Ile, Leu, and Val, as well as ornithine and citrulline (Clark et al., 1978).

The extraction efficiency of the mammary gland varies with individual AA. The mammary gland must do this to maximize milk protein output. For example, the concentration of Met and Lys relative to the total concentration of EAA in plasma is about half that found in milk protein (Clark et al., 1978). If the mammary gland extracted all the EAA equally, then protein output would be limited by the supply of Met and Lys. However, the mammary gland corrects for these inequalities by extracting Met and Lys to

a greater degree. Mackle et al. (2000) reported the extraction rate, calculated as arteriovenous difference/arterial concentration, of Lys and Met as 55.3% and 55.0% while extraction rates of the other EAA varied between 19% and 45%. These values are in agreement with those of Lescoat et al. (1996).

The mammary gland has the ability to vary its extraction rate in response to nutrient supply. Abomasal infusion of casein plus the BCAA into lactating cows reduced the extraction efficiency of Arg, Ile, Leu, Lys, and Val relative to the control treatment (Mackle et al., 2000). When an AA solution devoid of His was infused into the abomasum, mammary gland net fractional extraction of His increased about 5-fold, while for the remaining EAA, it was reduced approximately 2-fold (Bequette et al., 2000). As duodenal infusions of Met increased from 0 to 32 g/d, Met extraction rates fell from 58.3% down to 10.7% (Guinard and Rulquin, 1995). However, mammary uptake remained unchanged due to the increase in arterial Met concentrations. The mammary uptake to output ratio also was unchanged. These results demonstrate that the mammary gland varies its extraction rate to provide adequate AA supplies for milk protein synthesis.

1.12.3 Protein turnover in the mammary gland

In addition to milk proteins, the mammary gland also synthesizes constitutive proteins, such as structural proteins and enzymes. There is a substantial turnover rate of these proteins, such that in the goat, their turnover contributes 42% to 72% of the total protein synthesized in the mammary gland (Bequette et al., 1998). This turnover comes at an energetic cost to the mammary gland. If it is assumed that there is 1.55 to 2.6 g of total protein synthesized for every 1 g of milk protein secreted, the inclusion of protein

turnover increases ATP usage from 27 - 79 mol to 67 – 119 mol for every 0.95 kg of milk protein produced (Bequette et al., 1998). While protein turnover is advantageous in that it supplies the mammary gland with a steady supply of AA, it may also be detrimental in that it may compete for AA and energetic precursors for casein synthesis (Bequette and Backwell, 1997).

Milk proteins, primarily casein, are also degraded. The protease responsible for casein degradation is plasmin, which is found in milk in the active form and in the inactive form as plasminogen (Prosser et al., 1995). The caseins most susceptible to degradation are α_{s1}-casein and β-casein (Andrews, 1983). These are degraded in both the nascent and mature forms. Degradation of the nascent form involves cleavage to remove signal sequences, while in the mature molecule, it is proposed to occur in response to incomplete milk removal (Bequette and Backwell, 1997). Wilde et al. (1989) demonstrated that in mammary gland explants prepared from goats that were incompletely milked, intracellular degradation of casein occurred during secretion, while in the explants prepared from goats milked normally, casein degradation was not detected. The increase in degradation resulted from a reduction in secretory efficiency, which occurred as a result of the greater volume of residual milk. The authors proposed that the reduced secretory efficiency and increased casein degradation occurred through the action of a secreted milk constituent, which acted as a feedback inhibitor of milk secretion.

The rate of casein degradation appears to vary with factors such as feed restriction, milking frequency, and stage of lactation. In cows that were subjected to feed restriction of 45% for 3 wk, plasmin activity increased by 250% (Prosser et al., 1995). Plasmin

activity also increases in cows that are milked once daily compared to those milked twice daily (Stelwagen et al., 1994), and the ratio of plasminogen to plasmin decreases, indicative of more active enzyme. Plasmin activity is positively correlated with DIM; this effect is independent of the decrease in milk yield with advancing lactation, and may be associated with mammary involution (Politis et al., 1989)

1.12.4 Limiting amino acids for milk protein synthesis

Milk protein synthesis may be limited by several factors, the most obvious being the supply of AA. Three classes of AA may be limiting: EAA whose uptake by the mammary gland equals their output in milk protein because these AA typically have high extraction rates; EAA whose uptake exceeds their milk output because they have an obligatory role within the gland other than milk protein synthesis; and NEAA, because their rates of synthesis may be inadequate to support high levels of milk protein synthesis (Mepham, 1982). The possibility exists that one AA is limiting, or that several are co-limiting. Early studies by Schwab et al. (1976), in which the change in milk protein secretion was measured in response to AA infusions, indicated that Lys and Met were first and second limiting, or co-limiting, for protein secretion in cows fed corn-based diets. Subsequent studies have supported this finding (King et al., 1991; Polan et al., 1991; Schwab et al., 1992). While Lys and Met appear limiting in corn-based diets, that does not seem to be the case in grass- or alfalfa-based diets. Varvikko et al. (1999) infused cows that were fed grass silage-based diets with either Met or Lys, and saw no improvements in milk yield or milk protein content. In cows fed grass silage-based diets, milk protein yields were increased by His infusions (Vanhatalo et al., 1999). No further

improvements were seen when Lys and Met were infused in conjunction with His. Kim et al. (1999) also determined that His is first limiting when grass silage is fed.

In an attempt to alleviate AA deficiencies and increase milk protein yield, casein or free AA infusions have been used in lactating dairy cows. These infusions have resulted in variable, and often disappointing, milk protein responses (Clark, 1975). This may indicate that specific AA are still limiting, or that a limitation of other metabolites exists, particularly energy precursors. Milk protein synthesis consumes 10 to 12% of the mammary gland's ATP supply (Hanigan et al., 2001). If constitutive protein synthesis and protein turnover are considered, then 35% of the ATP supply may be used for protein synthesis (Hanigan et al., 2001). Supplementation with energy precursors has resulted in increased milk protein yields in several studies (Thomas and Chamberlain, 1984; DePeters and Cant, 1992; Hurtaud et al., 2000) but not all (Hurtaud et al., 1998). The increased yield may be the result of amino acid sparing, changes in endocrine status, or improvements in the energy status of the mammary gland. Composition of the basal diet and stage of lactation also influence the response to supplemental AA. Rulquin et al. (1994) reported that the milk protein response to rumen-protected Met and Lys was greater (29 g/d) in heifers fed a 15% CP diet compared to the response (15 g/d) in heifers fed a 13% diet. However, as the authors noted, AA other than Met and Lys may have been limiting in the low protein diet, and therefore limited the milk protein response. Casein infusions (1000 g/d) resulted in a greater increase in milk protein yield (28%) in cows in their first month of lactation compared to the increase (17%) in cows in their 7th month of lactation (Istasse et al., 1986). The effect of stage of lactation may be the result of changes in hormone sensitivity, changes in tissue depletion and repletion, and changes

in AA uptake by the mammary gland (Bequette et al., 1998). Other factors that may limit the milk protein response include endocrine status of the animal, genetic potential of the animal, environmental factors, and management strategies.

1.13 Conclusion

The transition period for the dairy cow is the most critical period of the lactation cycle. Dry matter intake often falls sharply in multiparity cows, endocrine status changes substantially from the prepartum to the postpartum period, and large nutrient demands are placed on the cow with the onset of lactation. Depending on the energy density of the diet and the extent of DMI reduction, the cow may actually enter into negative energy balance before calving. The decline in feed intake, in association with changes in hormonal status, causes an elevation in plasma NEFA concentrations, which in turn leads to fat deposition in the liver. Depending on the extent of the lipid infiltration, severe fatty liver may develop, which then has negative consequences on liver function and the overall health of the cow.

Similarly, low protein intakes may also induce body protein mobilization prepartum, which then accelerates postpartum. In the prepartum period, the mobilized AA are extensively used by the fetus as an energy source. Postpartum, AA that are released from peripheral tissues are used by the mammary gland for constitutive and milk protein synthesis. One of the goals then in the prepartum period is to ensure that the cow maintains adequate energy and protein intake so that excessive body lipid and protein mobilization can be avoided.

Although ensuring adequate protein intake is important, it is the balance of AA supplied to the cow that is critical. A deficiency of only one AA relative to the others can

impede protein synthesis and result in the oxidative catabolism of the remaining AA. The AA requirements of the cow, both in the dry and lactating period, are not well defined. Studies over the years have implicated Met and Lys as being most limiting when corn-based diets are fed, but the extent and order of limitation is dependent on the composition of the diet. Thus, prediction of limitation is very difficult for particular diets and feeding situations.

Several models are available that predict AA supply and requirements for dairy cows. These prediction approaches base AA supply on the amount of digestible AA at the duodenum. Although this approach has merit, it fails to take into account modulation of AA supply by splanchnic tissues. Accurate prediction of postsplanchnic AA availability would certainly be beneficial to accuracy of prediction of milk protein responses to dietary protein supplementation.

1.14 Objectives

The objectives of this thesis fall into three broad categories:

1. To determine if prepartum dietary energy and protein densities higher than that recommended by NRC (1989) are beneficial to the transition dairy cow. Indices of performance include:

a) milk production and composition
b) incidence of metabolic disorders
c) body weight and body condition score
d) plasma metabolite profiles
e) hepatic TG and glycogen content
f) abundance and activity of lipogenic enzymes

2. To determine plasma concentrations of AA during the periparturient period. Very few studies have reported plasma AA concentrations in the transition cow. This

information may be valuable when used in association with other parameters as an indicator of AA limitation.

3. To predict milk protein yield and the milk protein response to AA supplementation by generating a database of available studies in which AA have been infused in lactating cows, and developing regression equations that take into account factors such as AA composition of the basal diet, AA composition of the infusion, energy intake, BW, DIM, parity, and other factors.

Table 1-1 CNCPS (2000) efficiency values for absorbed amino acid use.

	Maintenance	Pregnancy	Lactation
Arginine	0.85	0.38	0.35
Histidine	0.85	0.32	0.96
Isoleucine	0.66	0.32	0.66
Leucine	0.66	0.42	0.72
Lysine	0.85	0.53	0.82
Methionine	0.85	0.35	1.00
Phenylalanine	0.85	0.48	0.98
Threonine	0.85	0.57	0.78
Valine	0.66	0.32	0.62

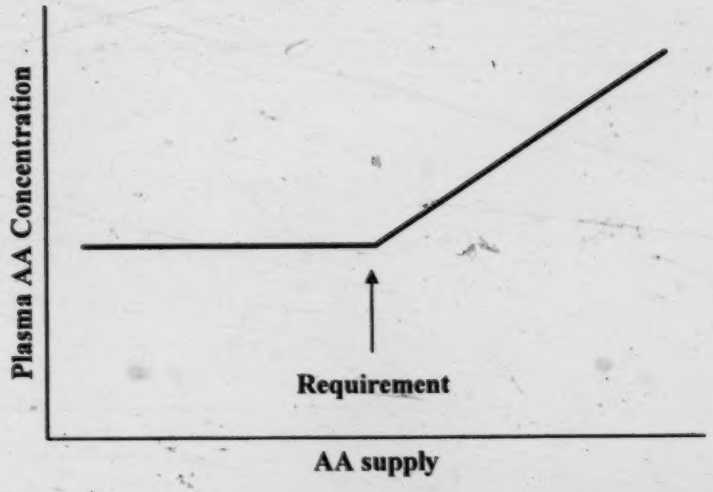

Figure 1-1 Two-phase broken line plasma amino acid response curve. Below the animal's requirement plasma AA concentration remains unchanged as AA supply increases. Above the animal's requirement, plasma concentration increases as supply increases.

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CHAPTER 2

Peripartum Performance and Metabolism of Multiparous Dairy Cows in Response to Prepartum Energy and Protein Intake

2.1 Introduction

The transition period for dairy cows, defined as 3 wk precalving to 3 wk postcalving (Grummer, 1995), is the most critical phase of the lactation cycle. This period is characterized by a substantial decline in feed intake (Bertics et al., 1992), lipid mobilization leading to elevated plasma NEFA and hepatic TG content (Vázquez-Añón et al., 1994), protein mobilization as evidenced by increased urinary 3-methylhistidine:creatinine ratios (VandeHaar et al., 1999), and endocrinological changes in support of parturition and lactogenesis (Grummer, 1993). When feed intake is reduced, dependence on lipid stores increases with a subsequent increase in the probability of hepatic lipidosis and metabolic disorders developing (Goff and Horst, 1997). Increasing the nutrient density of the diet may offset the negative effects of reduced feed intake before parturition. Feeding prepartum diets that are more energy dense than that recommended by NRC (1989) has led to reductions in both plasma NEFA concentrations and lipid infiltration of the liver (VandeHaar et al., 1999). Similarly, when prepartum diets containing higher levels of protein than that recommended by NRC (1989) have been fed, there have been pre- and postpartum improvements in metabolic status of the cows (Putnam and Varga, 1998) and body condition score (Van Saun et al., 1993). However, in the majority of studies when prepartum diet nutrient density has been increased, there has been little or no response in milk production (Putnam and Varga, 1998; Huyler et al., 1999; Putnam et al., 1999). In most cases where effects of prepartum dietary protein level have been examined, energy density of the diet has been kept

constant; perhaps energy limitations are preventing potentially beneficial effects of additional protein from being expressed. A similar situation may exist when energy supply is increased but protein content is held constant. Consequently, our objective was to examine effects of prepartum diets with two energy densities and two protein densities, in a factorial arrangement, on lipid and protein metabolism as well as lactation performance.

2.2 Materials and Methods

2.2.1 Animals and Treatments

Twenty-eight Holstein cows entering their 2nd or 3rd lactation were used. Cows were blocked by parity, milk production, and expected calving date, and were randomly assigned within each block to one of four dietary treatments – high energy-high protein (HEHP), high energy-low protein (HELP), low energy-high protein (LEHP), and low energy-low protein (LELP). The four dietary treatments were arranged as a 2 × 2 factorial, with 2 levels of energy, 1.65 Mcal/kg NE_L (HE) and 1.30 Mcal/kg NE_L (LE) and two levels of crude protein, 17.0% (HP) and 12.5% (LP). Ingredient and nutrient composition of the diets is in Table 2-1. Forage NE_L content was calculated from Minnesota Valley Testing Laboratories: [1.044-(0.0123 × ADF)] × 2.2046 for legumes, [1.085-(0.015 × ADF)] × 2.2046 for grasses, and 2.072-(0.0176 × ADF) for whole crop barley silage, where NE_L density is in Mcal/kg, and ADF is acid detergent fibre in percentage of DM. The NE_L content of the concentrates was calculated from NRC (1989) values for the ingredients. Based on an estimated DMI of 13 kg/d, and using NRC (2001) equations, NE_L density of the diets was 1.66, 1.60, 1.39, and 1.33 Mcal/kg DM for HEHP, HELP, LEHP, and LELP, respectively. Diets were formulated using the Cornell

Net Carbohydrate and Protein System (CNCPS; O'Connor et al., 1993) to supply similar amounts of metabolizable protein from the two HP diets and from the two LP diets. Based on 13 kg/d DMI, metabolizable protein supply was calculated (NRC, 2001) to be 1466, 1017, 1523, and 1061 g/d for HEHP, HELP, LEHP, and LELP, respectively.

Cows were purchased for this study in late lactation (>280 DIM). While lactating, all the cows were fed a late lactation diet consisting of (DM basis) 11.8% alfalfa hay, 16.8% alfalfa silage, 26.4% whole crop barley silage, and 45% rolled barley-based concentrate mix. In an attempt to equalize BCS by the time of treatment initiation, cows with a BCS < 3.25 (scale of 1 to 5) were fed additional concentrates (1 to 2.5 kg/d, depending on BCS). The equalization of BCS continued during the dry period. Cows with a BCS > 3.25 were fed a far-off dry diet consisting of (DM basis) 16.9% grass hay, 15.9% alfalfa silage, 48.7% whole crop barley silage, and 18.5% concentrate mix. Cows with a BCS < 3.25 continued on the late lactation diet but did not continue to receive additional concentrates. They were switched to the far-off dry diet when they reached BCS 3.25, or at a minimum of 2 wk before starting on the dietary treatments. At the time of treatment initiation, BCS of the cows was 2.96, 3.21, 3.25, and 3.25 for HEHP, HELP, LEHP, and LELP, respectively (SEM = 0.12). Adaptation to the dietary treatments occurred over a 3-d period with the proportion of the experimental diet relative to the far-off dry diet increased by 25%/d. Cows were then fed the dietary treatments from 21 d before expected calving until the day of calving. Due to inherent errors in predicting calving dates, the length of time on treatment ranged from 12 to 31 d, with a mean of 21.3 ± 1.2 (SEM). The number of cows on treatments for less than 14 d was 1, 1, 0, and 0 for HEHP, HELP, LEHP, and LELP, respectively. Cows were housed in tie-stalls with free

access to water, and exercised daily from ~ 0930 to 1030. All animal procedures were approved by the Faculty of Agriculture, Forestry, and Home Economics Animal Policy and Welfare Committee in accordance with the guidelines of the Canadian Council on Animal Care (1993).

Prepartum, diets were fed ad libitum as a total mixed ration (TMR) once a day at ~ 0700 h. After calving, cows were fed a common TMR formulated to meet NRC (1989) recommendations (Table 2-2). The TMR was offered ad libitum once daily at ~ 0800. Throughout the trial, i.e., 21 d before expected calving to 42 DIM, amounts of feed offered were adjusted daily to maintain 10% orts, and DMI was recorded daily. Chromic oxide (10 g/cow/d) was mixed into the diets as an indigestible marker for estimating total tract indigestibility of nutrients.

Moisture content of the silages was determined weekly and used to make ration adjustments. All diet ingredients were sampled weekly and composited bi-weekly. Samples were dried in a forced air oven at 60°C for 48 h and ground to pass a 1 mm screen. For determination of DM, samples were dried at 100°C for 24 h, and OM was calculated as the weight lost upon ashing for a minimum of 6 h at 550°C. Orts were sampled twice weekly and composited by week for determination of DM. For analytical procedures, orts were composited by cow within period, i.e., precalving and postcalving. Ingredients and orts were analyzed for CP (nitrogen x 6.25) using a Leco FP-428 Nitrogen Determinator (Leco Corporation, St. Joseph, MI). The ADF and NDF were determined as described by Van Soest et al. (1991). Mineral content was determined by Inductively Coupled Plasma Emission spectroscopy (JY70 plus inductively coupled argon plasma; Jobin Yvon, Division d'instruments S.A., Longjumeau, France).

Cows were weighed and body condition scored weekly at ~ 1030 h throughout the trial, and on d 25 (pretreatment) before expected calving and d 1 after calving. Energy balance was calculated as the difference between energy consumed and required, where NE required prepartum = maintenance + pregnancy, and NE required postpartum = maintenance + milk. Because this study was completed before the release of the NRC (2001), and formulation of the diets and calculation of the energy content was based on NRC (1989) values, energy requirements are also based on NRC (1989). Energy required for maintenance (Mcal/d) = 0.08 × kg $BW^{0.75}$ for 3rd lactation cows and 1.1(0.08 × kg $BW^{0.75}$) for 2nd lactation cows, NE for pregnancy (Mcal/d) = 0.024 × kg $BW^{0.75}$, and NE for lactation (Mcal/kg milk) = 0.3512 + (0.0962 × %fat). Crude protein balance was calculated according to NRC (1989). At calving, the calf's sex and weight were determined prior to colostrum feeding.

Cows were milked at 0600 and 1800. Milk yield was electronically recorded for the first 42 d of lactation. Milk samples, taken twice weekly (Monday pm/Tuesday am and Thursday pm/Friday am) were analyzed for CP, fat, and lactose by infrared analysis at the Alberta Central Milk Testing Laboratory (Edmonton, AB, Canada). Milk samples from wk 2 and 6 were analyzed for FA composition. Milk fat was extracted and the FA profiles measured by gas chromatography using a Varian model 3700 with flame ionization detector (Khorasani et al., 1991).

2.2.2 Sampling and Analysis

Blood samples were collected via the coccygeal vein at ~ 4 h after feeding on two consecutive days prior to starting on the dietary treatments, once within 24 h of calving, and weekly from initiation of the trial until 28 DIM. Additional samples were collected

at 10, 17, and 24 DIM for progesterone analysis. Blood was collected into evacuated tubes containing sodium heparin, EDTA, potassium oxalate and sodium fluoride, or no additives. All tubes for plasma collection were immediately placed on ice and centrifuged within 4 h. Samples for serum were allowed to clot at room temperature for at least 4 h before centrifugation. Samples were centrifuged at 4°C for 15 min at 3300 × g, and stored at -70°C until analysis. Nonesterified fatty acids in serum were determined using the NEFA-C kit (Wako Chemicals USA Inc., Richmond, VA) with the modifications of Johnson and Peters (1993). Plasma BHBA was determined by the procedure of Williamson and Mellanby (1974) adapted to a 96-well microliter plate format. Plasma glucose was determined using the Beckman Glucose Analyzer 2 (Beckman Instruments, Missisauga, ON). Insulin and IGF-1 (Lapierre et al., 2000a) and GH (Lapierre et al., 2000b) were analyzed by double antibody radioimmunoassay. Intra- and interassay coefficients of variation were 9.1% and 6.6%, <1% and 8.4%, and <1% and <1% for insulin, IGF-1, and GH, respectively. Plasma 3-MH was determined by HPLC as described by Zurek et al. (1995). Plasma amino acids were quantified by HPLC using the procedure of Sedgwick et al. (1991). Plasma samples were also analyzed for urea nitrogen (PUN; Sigma BUN kit 535, Sigma Chemical Co., St. Louis, MO) and progesterone (Coat-A-Count®; Diagnostic Products Corporation, Los Angeles, CA). Cows were considered to have reached first estrus on the first of 3 consecutive samples with a progesterone concentration greater than 1.0 ng/ml (Simmons et al., 1994).

Liver samples were obtained by biopsy (Smart, 1985) on d 25 before expected calving, and on d 1 and 21 postpartum. The liver tissue (~1 g) was rinsed in phosphate buffered saline to remove excess blood and immediately placed in liquid nitrogen.

Samples were stored at –30°C until analyzed for TG and glycogen content. Liver lipids were extracted by the method of Folch et al. (1957) and TG content determined by the method of Fletcher (1968) as modified by Foster and Dunn (1973). Liver glycogen was determined by the method of Lo et al. (1970).

Urine samples were collected on d -26, -7, 10, and 20 relative to calving. Samples (500 ml) were collected at 1100 h and stored at –20°C until analyzed for creatinine (Sigma kit 555, Sigma Chemical Co., St. Louis, MO) and 3-MH as described by Zurek et al. (1995).

To determine nutrient digestibilities, rectal grab samples of feces were obtained twice daily at 12 h intervals for 6 d during d –14 to d –9 and d 8 to d 13 relative to calving. In both the prepartum and postpartum periods, the fecal collection began after the cows were on the chromic oxide-marked feed for 7 d. Each day the samples were obtained 2 hours later than on the previous day. At each collection, 250 g were retained and composited within period, i.e., precalving and postcalving. Samples were stored at -20°C until analyzed. Chromium content in feces and feed was analyzed by atomic absorption spectrophotometry as described by Fenton and Fenton (1979). Nutrient digestibilities were determined from the ratios of Cr concentrations in the diet to those in feces. Fecal samples were analyzed for CP, ADF, NDF, and OM using the same methods as those described for the feed ingredients.

Subcutaneous adipose tissue was biopsied from the perianal region on d 25 before expected calving and on d 1 and 21 postpartum. Tissue was sampled from the left side on d 1, and from the right side on d –25 and d 21. Adipose tissue (~10 g) was rinsed in

phosphate buffered saline to remove excess blood, immediately placed in liquid nitrogen, and stored at -70°C until analysis for western blotting and enzyme activity.

Western blotting was performed for ACC and FAS. Homogenization of the adipose tissue (~1.0 g) and collection of the supernatant was performed as outlined by Moibi et al. (2000). Supernatants were aliquoted and stored at -70°C until analysis. Proteins were quantified with a BCA Protein Assay Reagent kit (Pierce, Rockford, IL). Proteins were separated using SDS-PAGE and quantified as described by Beswick and Kennelly (1998). Twenty-five µg of protein were loaded on the gel. The ACC was detected using streptavidin conjugated to horseradish peroxidase at a concentration of 1:500. The FAS was detected with a primary mouse anti-fatty acid synthase monoclonal antibody and a secondary goat anti-mouse-horseradish-peroxidase. The primary antibody concentration was 1:250 and the secondary antibody was used at a concentration of 1:500.

To measure enzyme activity, 10% PEG was added to one aliquot and the sample centrifuged at 11,000 × g for 10 min at 4°C. The pellet was resuspended in 200 µl of resuspension buffer (100 mM Tris HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM Na pyrophosphate, and 4 µg/ml aprotinin, leupeptin, and pepstatin A) and stored at -70°C. The protein content was determined by the bicinchoninic acid method (Pierce, Rockford, IL). The $H^{14}CO_3^-$ fixation method as described by Moibi et al. (2000) was used to assay ACC activity. Acetyl-CoA carboxylase activity is expressed as nmol of ^{14}C-bicarbonate incorporated into malonyl-CoA/min per mg protein. The method of Moibi et al. (2000) was used to assay FAS activity. Activity is expressed as nmol NADPH/min per mg protein. Lipoprotein lipase activity was assayed according to Liesman et al. (1995). Activity is expressed as nmol oleic acid/h per mg protein.

2.2.3 Statistical Analysis

One cow on the HELP treatment aborted before the trial began and one cow on the LEHP treatment calved the day after going on treatment, thus 26 cows were used for the trial. One cow on the HELP diet died two days after calving due to calving difficulties, therefore, measurements from the postpartum period were from 25 cows. Dry matter intake data was excluded from one cow on the LELP treatment due to excessive feed wastage. One cow on the LELP diet developed a right displaced abomasum shortly after parturition, and data during the recovery period (one week) was excluded. The cow was successfully treated surgically and continued on the trial. Two cows, one each on HEHP and LELP treatments, developed footrot after calving that subsequently affected their DMI, so data from the first week postpartum were excluded. Milk compositional data from the first three DIM were excluded for all cows.

Repeated measures data were analyzed using the MIXED procedure of SAS (1999b). The statistical model included block, time, energy, protein, energy by protein interaction, energy by time interaction, protein by time interaction, and energy by protein by time interaction. For DMI, the periods tested were precalving vs. wk 1 to 3 vs. wk 4 to 6 postcalving. For milk yield, the periods were wk 1 to 3 and wk 4 to 6 postcalving. For each analyzed variable with equal time spacing between samples, cow nested within energy by protein by block was subjected to three covariance structures: autoregressive order 1, compound symmetry, and spatial power law. For variables with unequal spacing, only compound symmetry and spatial power law covariance structures were used. The covariance structure that resulted in the largest Schwarz's Bayesian Criterion and Akaike's Information Criterion was used. Preplanned comparisons for the effect of

time were analyzed by PDIFF (SAS, 1999a). Body weight, plasma insulin and IGF-1 concentrations, and ACC, FAS, and LPL protein abundance and activity before the initiation of treatments were significantly different among the different treatments and so pretreatment data were used as covariates. Single measurement data were analyzed using PROC MIXED with block, energy, protein, and the energy by protein interaction in the model. Progesterone was analyzed as categorical data using PROC FREQ (SAS, 1999a). Correlation coefficients were determined using PROC CORR (SAS, 1999a). Data are reported as least squares means with pooled standard errors. In the figures, wk -3 represents data obtained from d -15 to d -21, wk -2 represents data from d -8 to d -14, and wk -1 data are from d -1 to d -7. Pretreatment values are not shown. Significance was declared at $P < 0.05$.

2.3 Results

2.3.1 Metabolic Parameters

Dietary protein had a significant effect on BHBA concentrations (12.8 vs. 10.6 mg/dl for HP and LP; $P = 0.03$; Figure 2-1), however, this effect was evident only in the postpartum period, primarily at wk 1 (protein × week interaction, $P = 0.10$). Time also influenced BHBA concentrations, with levels being higher postpartum than prepartum ($P < 0.001$). Across all time points, glucose was unaffected by prepartum treatment (Figure 2-2). As with BHBA, the effect of time was significant ($P < 0.001$); glucose concentrations were lower postcalving than precalving.

Plasma insulin concentrations declined steadily from wk -3 to calving and then remained at low levels postpartum (Figure 2-3). There was a tendency for an energy by protein interaction ($P = 0.06$). When HE was associated with HP, insulin was higher than

when it was associated with LP. The reverse was true for LE with insulin concentrations higher when LE was associated with LP. The concentrations of IGF-1 over time followed the same general pattern as for insulin (Figure 2-4), however there were no treatment effects. From wk -3 to wk -1, plasma GH concentrations were relatively stable and then increased rapidly ($P < 0.001$) as calving approached (Figure 2-4). Concentrations peaked at 1 wk postcalving and declined thereafter. There were no treatment effects either prepartum or postpartum.

2.3.2 Lipid Metabolism

There was an energy by protein by week interaction for plasma NEFA ($P = 0.005$; Figure 2-5). Concentrations of NEFA were low in the prepartum period (214.9 ± 32.1 μmol/L) and peaked at calving for all dietary treatments except HEHP, which peaked at 1 wk postcalving. Cows fed LEHP had the highest NEFA levels at calving (1286.1 ± 90.2 μmol/L). Postcalving, the concentration of NEFA declined as DIM increased and did not differ among the treatments, averaging 422.0 ± 27.2 μmol/L. There was an energy by week interaction ($P < 0.001$); at calving, cows fed the LE diets had higher NEFA concentrations than cows on the HE diets, but at other time points, there were no differences between the two groups. There was a negative correlation between the change in NEFA from d -7 to calving and the change in DMI during the same time period ($r = -0.59$, $P = 0.002$). Because reductions in DMI lead to reductions in energy balance, there was also a negative correlation between NEFA concentrations and energy balance during the last week prepartum ($r = -0.72$, $P < 0.001$) and at calving ($r = -0.60$, $P = 0.002$).

Liver TG content increased approximately 5 fold from 25 d precalving to the day of calving, and another 1.5 fold from calving to 21 d postcalving (time effect, $P < 0.001$; Figure 2-6). Energy content of the diet had a significant effect on TG concentrations, which averaged 7.1% for HE and 9.8% for LE ($P = 0.06$). There was a negative correlation between liver TG and energy balance on the day of calving ($r = -0.50$, $P = 0.01$). Liver glycogen content was 14.4% at d -25, declined to 4.9% on d 1, and then increased to 6.7% on d 21 (time effect, $P < 0.001$; Figure 2-6). Prepartum treatment had no effect on hepatic glycogen content. There was a negative relationship between liver TG and glycogen content ($r = -0.70$, $P < 0.001$).

Western blot analysis of the adipose tissue revealed that there was an effect of treatment on the abundance of the ACC protein (Table 2-3, Figure 2-7). Acetyl-CoA carboxylase abundance was higher with the HE treatments on d 1 postcalving compared to the LE treatments, but was the same on d 21 (energy × day interaction, $P = 0.002$). There was also an energy by day effect on the abundance of FAS ($P = 0.006$). On d 1, the FAS abundance in adipose tissue averaged 0.95 OD × mm^2 in cows on the HE treatments and was significantly higher than the abundance in cows on the LE treatment, which was 0.51 OD × mm^2. On d 21 there were no differences between the two treatments. The effect of time was significant ($P < 0.001$) for both ACC and FAS, with values lower on d 21 vs. d 1. Prepartum dietary treatment had no effect on ACC activity on d 1 or d 21 postcalving (Table 2-4). As with ACC abundance, the effect of time was significant ($P = 0.05$) for ACC activity. Fatty acid synthase activity was not influenced by treatment, but did decline from d 1 to d 21 (time effect, $P = 0.06$). Only time was a

significant factor on the activity of lipoprotein lipase, with the activity decreasing (P = 0.004) as lactation progressed.

2.3.3 Protein Metabolism

In the precalving period, PUN was higher for the HP treatments vs. LP (15.9 vs. 11.5 mg/dl), but this effect disappeared postcalving (protein × time effect, P = 0.003; Figure 2-8). There was also a protein by energy interaction (P = 0.05). When HE was associated with HP, PUN was higher than when HE was associated with LP (16.8 vs. 13.3 mg/dl). However, PUN was similar on the LE diets regardless of dietary protein level (14.3 mg/dl for LEHP vs. 13.7 mg/dl for LELP). A protein by time interaction also existed for plasma 3-MH. 3-methylhistidine was higher precalving for cows fed LP compared to those fed HP (P = 0.004; Figure 2-9). The concentration of 3-MH peaked at calving for cows on the LP treatment, whereas it peaked at 1 wk postcalving for cows on the HP treatment. In the postpartum period, there was no effect of treatment on 3-MH.

Plasma AA concentrations are shown in Table 2-5. Because there were no treatment effects for the majority of the AA, data are pooled and presented by day of sampling only. With the exception of His, Met, and Phe, all of the EAA concentrations decreased at calving (P < 0.001). Relative to d 1, the concentrations at d 21 were higher for Arg, Ile, Lys, Thr, and Trp, lower for His and Phe, and unchanged for Leu, Met, and Val. Arginine, Ile, Lys, Met, Phe, Thr, and Trp were unaffected by treatment. Over the course of the trial, histidine concentrations were affected by energy content of the diet (50.5 μmol/L vs. 55.5 μmol/L for HE and LE, respectively, P = 0.03). Leucine concentrations were increased by the HP diet (130.9 μmol/L and 108.8 μmol/L for HP and LP, respectively). Valine concentrations were also influenced by the protein content

of the diet ($P = 0.02$), and were higher for the HP treatments (198.2 μmol/L) than the LP treatments (172.1μmol/L). The majority of the NEAA were unaffected by diet. Glutamine and Gly concentrations increased sharply between d -7 and d -2. At calving, the concentration of Glu rose and the concentrations of Ala and Ser were unchanged. The other NEAA experienced a drop at calving. With the exception of Gln, which was lower, and Ala, Gly, and Ser, which were unchanged, concentrations of the NEAA were higher on d 21 postcalving vs. d 1.

Across all time points, urinary creatinine concentration was unaffected by treatment. Average values were 97.7, 141.1, 97.3, and 77.8 mg/dl on d -25, -7, 10, and 20 relative to calving, respectively. Average creatinine concentrations were lower ($P < 0.001$) postpartum than during the prepartum treatment period. The 3-MH:creatinine ratio increased from d -25 to d -7, and was subject to an energy by time interaction ($P = 0.1$; Figure 2-10). On d -25, -7, and 10 there were no treatment differences, but on d 20, the ratio was higher for cows fed the HE diets compared to those fed LE.

2.3.4 DMI, BW, BCS, and Energy and Protein Balance

Prepartum DMI decreased as calving approached, and averaged 13.8 kg at 3 wk prepartum and 7.6 kg on the day of calving (Figure 2-11). The reduction in DMI from d -7 to the day of calving was 26.5%, 33.4%, 60.0%, and 35.1% for HEHP, HELP, LEHP, and LELP, respectively. The reduction of the LEHP treatment was greater ($P < 0.05$) than that of the other treatments. There was an energy by period interaction on DMI ($P = 0.01$). During the precalving period, DMI of HE cows was the same as LE cows, but during the postcalving period, intake was higher for HE than LE (Table 2-6). The same interaction existed for DMI as a percentage of BW ($P < 0.01$; data not shown). A protein

by period interaction ($P < 0.001$) indicated that prepartum CP intake was higher for HP than LP (2.68 vs. 2.19 ± 0.09 kg/d), but postpartum CP intake was not different between the two treatments. During both the prepartum and postpartum periods, net energy intake was higher ($P = 0.01$) for cows fed HE compared to those fed LE (prepartum: 20.7 vs. 17.2 ± 1.0 Mcal/d; postpartum: 30.4 vs. 26.8 ± 1.0 Mcal/d).

Prepartum, cows were in positive energy balance until the immediate precalving period, entering a period of negative energy balance at d 1, -1, -3, and -2 for HEHP, HELP, LEHP, and LELP, respectively (Figure 2-12). Cows on the LEHP treatment experienced the largest drop in DMI at calving and therefore were in the most negative energy balance. After calving, cows on all four treatments were in negative energy balance, reaching a nadir at wk 1 postpartum. Across all weeks, cows on HE were in less negative ($P = 0.003$) energy balance than cows on LE. Crude protein balance was affected by treatment (protein × week interaction, $P < 0.001$; Figure 2-13). During the prepartum period, cows on the HP treatments were in significantly greater protein balance than cows on LP, while there was no difference between the two groups postpartum.

Prepartum BW and BW changes were not affected by treatment (Figure 2-14, Table 2-6). There was also no effect of treatment on postpartum BW and BW change. Cows lost on average 55.2 ± 9.6 kg during the first 42 DIM. Body condition score and BCS changes in the prepartum and postpartum periods were unaffected by treatment (Table 2-6). Over the first 42 DIM all cows lost condition, with the average loss being 0.54 ± 0.09 units.

Average calf weight at birth (44.8 ± 1.6 kg) was unaffected by treatment. The incidence of metabolic disorders was relatively low. One cow fed HEHP developed

mastitis, one cow on the HELP treatment had dystocia and subsequently died, two cows fed LEHP experienced retained placenta, and one cow on the LELP treatment had a right displaced abomasum. Prepartum treatment had no effect on day to first estrus as measured by plasma progesterone concentration. Eleven of the 25 cows reached first estrus by 28 d postpartum.

2.3.5 Milk Yield and Composition

Treatment had no effect on milk yield during the first 42 DIM (Table 2-6, Figure 2-15). Because of difficulties with the adipose tissue biopsies at d 21 that subsequently affected DMI and milk yield, milk production was analyzed by period for the two 3-wk periods following calving. Milk yield averaged 32.3 ± 1.9 kg/d during the first 21 DIM and 40.2 ± 2.0 kg/d during d 22 to 42. Although cows fed the HE diets produced over 2 kg more milk per day than cows on the LE diets during the first 21 DIM this difference was not statistically significant (P = 0.22), likely due to inadequate replication. Completion of this study with a greater number of cows may have allowed detection of differences in performance. Milk fat, protein, and lactose yields were unaffected by diet (Table 2-6). Milk fat content during the first 3 wk postpartum tended to be lower with the HE diets than the LE diets, but was not different during wk 4 to 6 (energy × period interaction, P = 0.08). There was no effect of treatment on milk protein content. Milk FA composition at wk 2 and wk 6 was also unaffected by treatment, therefore, average data are presented in Table 2-7. There was a time effect ($P < 0.05$) for all the FA except C4:0, C16:0, C18:3, C20:0 and C22:1. At wk 2, the concentration of short chain FA was lower ($P < 0.001$) than at wk 6, while the long chain FA had higher concentrations ($P < 0.001$) at wk 2 compared to wk 6.

2.3.6 Nutrient Digestibility

Whole tract apparent nutrient digestibility is shown in Table 2-8. Prepartum DM ($P = 0.002$) and OM digestibility was higher ($P < 0.01$) for the HE diets than the LE diets (DM: 69.3% vs. 64.0%; OM: 72.6% vs. 66.8%). Crude protein digestibility was affected by dietary protein level ($P = 0.001$), averaging 69.6% for the HP treatments and 61.2% for LP. Neither energy nor protein content of the dietary treatments affected prepartum ADF digestibility, but there was an energy by protein interaction ($P = 0.05$). When HE was associated with HP, ADF digestibility was higher than when it was associated with LP, however, when LE was associated with HP, ADF digestibility was lower than when it was associated with LP. Neutral detergent fibre digestibility was higher for the HP diets (61.1%) than the LP diets (55.5%). There were no treatment effects on nutrient digestibility in the postpartum period.

2.4 Discussion

2.4.1 Lipid Metabolism and Metabolic Parameters

The gradual increase in plasma NEFA concentrations from wk -3 to wk -1 (Figure 2-5) has been suggested as a feed intake effect, while the rapid increase in the immediate precalving period is hormonally regulated (Grummer, 1993). Force-feeding cows during the transition period attenuated the rise in plasma NEFA at calving but did not completely eliminate it (Bertics et al., 1992), suggesting that hormonal control plays a role. Vázquez-Añón et al. (1994) suggested that the NEFA spike at calving was associated with the sharp reduction in feed intake and with the elevation in plasma lipolytic hormones that occurs in conjunction with the onset of calving. Data from the present study appear to support these hypotheses because the change in NEFA during the week

precalving was negatively correlated with the change in DMI, and NEFA concentrations during the week precalving and at calving were negatively correlated with energy balance. It is interesting to note that LEHP cows, which had the highest NEFA at calving, experienced the largest drop in intake precalving and were in the most negative energy balance at calving. The cow with the lowest intake at calving (2.9 kg) had the highest NEFA concentration (2172 μmol/L).

Plasma NEFA 1 to 2 d precalving are highly correlated with liver TG 1 d postcalving (VandeHaar et al., 1999). At high circulating concentrations, large amounts of NEFA entering the liver are esterified due to physiological limits to oxidation (Goff and Horst, 1997) and TG accumulate as a result of the liver's limited capacity to export TG as very low density lipoproteins (Grummer, 1993; Drackley, 1999). Accordingly, in the present study, lower liver TG content at d 1 (~ 40%) for HE treatment cows corresponded with lower NEFA. Because elevated hepatic lipid content is associated with an increased incidence of metabolic disorders (Grummer, 1993) feeding high energy diets prepartum may offer benefits in terms of reducing postparturient disorders. Hepatic TG content was higher on d 21 than d 1 indicating that lipolysis and the release of NEFA were ongoing. Lipolytic rates reported by McNamara and Hillers (1986) substantiate this finding. The increase in hepatic TG from d 1 to d 21 was less than from d -25 to d 1, likely due to the substantially reduced plasma NEFA concentrations seen by 2 wk postpartum. These low concentrations probably result from high NEFA extraction by the mammary gland for milk fat synthesis. Bell (1995) estimated that in the immediate postpartum period, approximately 50% of circulating NEFA are either oxidized or

incorporated into milk fat. Although liver TG concentrations were quite high, clinical signs of fatty liver or ketosis were not observed.

Liver glycogen concentration was not influenced by prepartum energy intake, which contrasts the findings of Minor et al. (1998), where cows that consumed more energy precalving had higher hepatic glycogen content. Those authors hypothesized that the elevated glycogen content was related to the higher plasma glucose concentrations. In our study, plasma glucose was not affected by treatment. If hepatic glycogen content is indicative of carbohydrate status, then the carbohydrate status of the cows was not influenced by prepartum diet, although it was negatively impacted by parturition and the onset of lactogenesis.

Hippen et al. (1999) demonstrated that the ratio of hepatic TG to glycogen increased from parturition to 21 d postpartum. This is likely a reflection of the energy balance of the cow. In early lactation, cows are in negative energy balance, and as a result, mobilize lipid reserves and deplete hepatic glycogen reserves. In our study there was a strong negative correlation between hepatic TG and glycogen. The TG:glycogen ratio increased from 0.08 on d -25 to 2.77 on d 1 and 3.72 on d 21. The ratio was numerically smaller for the HE treatments compared to the LE treatments as a result of the significantly lower TG content and numerically higher glycogen content of cows on the HE treatments on d 1. Drackley et al. (1992) have suggested that cows with a TG:glycogen ratio greater than 2:1 are susceptible to development of fatty liver and ketosis, while those with a ratio less than 2:1 are more resistant. This suggestion was based on observations of ketosis induction (KI) studies (Drackley et al., 1992; Veenhuizen et al., 1991) in which cows subjected to the KI protocol had pretreatment ratios of greater than 2:1, whereas control

cows had lower ratios. However, it is not known whether the control cows would have developed ketosis had they been subjected to the protocol. The TG:glycogen ratio may give an indication of the susceptibility of cows to develop ketosis following a KI protocol, but may not indicate a cow's susceptibility to spontaneous ketosis under normal feeding conditions.

The higher plasma BHBA concentrations (Figure 2-1) postpartum vs. prepartum are consistent with Vázquez-Añón et al. (1994). In contrast to plasma NEFA, BHBA concentrations were higher at wk 1 postpartum than at parturition. Perhaps this lag time exists because NEFA provide substrate for BHBA synthesis. Cows on the LEHP treatment, that had the highest NEFA at calving, had the numerically highest BHBA at wk 1. Cows fed the HP diets had elevated BHBA concentrations compared to cows fed LP. If the cow's priority for nutrients is the mammary gland, milk synthesis will occur if protein is available even when energy is limiting. It may be possible that cows fed the HP diets precalving had more mobilizable protein reserves and this promoted greater lipid mobilization. If lipolysis occurred at a rate that exceeded the liver's capacity to completely oxidize or esterify the NEFA, then BHBA concentrations would rise.

Glucose concentrations that peaked at calving and were lower postpartum than prepartum (Figure 2-2) are common (Vázquez-Añón et al., 1994; Dann et al., 1999). The peak at calving may be related to the release of glucocorticoids immediately before calving that stimulate glycogenolysis and gluconeogenesis (Vázquez-Añón et al., 1994). The decreased glucose concentrations postpartum are probably related to low DMI and the concomitant reduction in propionate absorption, along with increased glucose requirements for milk synthesis.

Over the course of the trial, insulin concentrations were lower when dietary energy and protein were uncoupled (HEHP > HELP; LELP > LEHP). With the HE diets, high concentrations of propionate would be expected. Propionate is an insulin secretagogue that promotes high levels of insulin. Because protein is also an insulin secretagogue, it is not surprising that the HEHP diets would result in elevated insulin concentrations relative to HELP. It is unknown why the LELP diet would result in higher insulin levels than the LEHP diet, but it may relate to the large decrease in feed intake at calving of LEHP cows.

Prepartum dietary treatments had no effect on the concentration of IGF-1 and GH, but the change in their profiles is indicative of homeorhetic adaptation during late pregnancy and lactation. The elevation in GH concentration as parturition approaches directs nutrients away from adipose tissue and toward tissues such as the fetus and mammary gland. The uncoupling of IGF-1 and GH is similar to that reported by VandeHaar et al. (1999) and Grum et al. (1996).

To our knowledge this is the first study that has examined ACC and FAS protein abundance in adipose tissue of the transition cow. The higher ACC and FAS abundance on d 1 in cows fed the HE diets prepartum vs. cows fed LE is probably a reflection of the cows' energy balance. Cows on the LE diets had already been in negative energy balance for 4 d while cows on the HE diets were only in negative energy balance for 1 d. On d 21 postpartum there were no differences in ACC protein abundance due to treatment. It seems clear that the need for energy substrates by the mammary gland supercedes effects of prepartum treatment. Values for ACC abundance at d 21 are similar to those reported by Beswick and Kennelly (1998) for cows in midlactation receiving GH or GH releasing factor. It is not surprising that the values would be similar since GH inhibits insulin-

stimulated lipogenesis and enhances lipolysis (Bauman and Currie, 1980). A similar situation is seen in early lactation since GH is elevated around calving, adipose tissue is insensitive to the actions of insulin, and lipogenesis is severely inhibited. McNamara and Hillers (1986) measured the rate of lipogenesis in adipose tissue slices and found that the rate at 2 wk postpartum was only 5% of the rate at 1 month prepartum.

The change in FAS protein abundance from d -25 to d 21 was similar to that of ACC and reflects reduced lipogenesis around parturition. The abundance of FAS on d 21 was only 7.8% of the value on d -25. This reduction in abundance coincides with the change in lipogenic rates reported by McNamara and Hillers (1986). Fatty acid synthase is regulated primarily at the level of transcription (Goodridge, 1986). Insulin stimulates FAS expression in adipocytes by increasing the transcription rate of the gene (Claycombe et al., 1998). Thus, the low levels of insulin in the periparturient period would account, in part, for the low FAS protein content postpartum. The elevated GH concentrations observed postpartum may also contribute to the reduced FAS protein abundance. Porcine FAS mRNA content was significantly reduced in adipose tissue by administration of GH (Mildner and Clarke, 1991).

The activities of ACC, FAS, and LPL were unaffected by treatment but were affected by physiological state (Table 2-4). The reduced activity of ACC on d 21, only 7% that at d –25, is probably mediated by GH and insulin. Rapid regulation of ACC occurs through covalent modification. The enzyme is active when dephosphorylated, and insulin is a potent stimulator of dephosphorylation. During the periparturient period, insulin concentrations are low and GH concentrations are high rendering insulin inactive and preventing it from activating ACC. Insulin also stimulates LPL (Faulconnier et al.,

1994), but because adipose tissue is insensitive to the action of insulin in early lactation LPL activity is reduced. NEFA are also thought to inhibit LPL activity (Fielding and Fielding, 1991). The reduction in FAS activity from the prepartum period to the postpartum period is consistent with the decline in FAS abundance.

2.4.2 Protein Metabolism

Higher prepartum PUN concentrations for cows fed the HP diets vs. those fed LP are similar to findings of Huyler et al. (1999) and Greenfield et al. (2000). Elevated PUN concentrations are indicative of increased ureagenesis, a result of oxidation of amino acids in excess of those required for protein synthesis, or of detoxification of absorbed ammonia (Lobley et al., 2000). The more positive protein balance of the cows on the HP treatments also indicates that protein, either rumen degradable or rumen escape, may have been supplied in excess of requirements for the majority of the prepartum period. As parturition approached, PUN declined consistent with the reduction in dietary protein intake.

The effect of dietary treatment on plasma 3-MH was opposite of that on PUN. Prepartum 3-MH concentration was higher for the LP cows compared to HP, and the peak in plasma 3-MH occurred at calving for the LP cows, but was at 1 wk postcalving for the HP cows (Figure 2-9). These results suggest differences in protein metabolism between the two groups since 3-MH is released upon protein degradation, primarily from skeletal muscle (Young and Munro, 1978). The higher concentration and earlier peak in the LP cows indicates that skeletal muscle protein was being degraded earlier in gestation and to a greater extent in the LP cows. Regardless of treatment, it appears that the greatest demand for AA, relative to supply, is at calving and the first week postpartum,

when the greatest negative protein balance also occurred. Postpartum, 3-MH concentrations were similar among the treatment groups, suggesting that there was no residual effect of prepartum dietary supply, and that mobilization of body stores was equal across treatments. It appears that greater body protein reserves prepartum does not allow for greater protein mobilization postpartum.

The ratio of urinary 3-MH to creatinine has been used as a measure of protein degradation standardized for muscle mass (Simmons et al., 1994). The ratio in the prepartum period was not altered by treatment suggesting that protein degradation was similar among the treatments. This contrasts the study of VandeHaar et al. (1999) where increasing the energy and protein content of the prepartum diet increased the 3-MH:creatinine ratio. Those authors suggested that the increased muscle protein degradation occurred in conjunction with increased protein synthesis, and that the more nutrient dense diet supported greater amounts of protein synthesis. Insulin and GH concentrations were not reported in that study, but that data may have helped to substantiate that speculation. The reduction in plasma insulin before calving in our study would not support the idea of increased muscle protein synthesis.

That the 3-MH:creatinine ratio was highest at 10 d postcalving and started to decline thereafter is consistent with Simmons et al. (1994). The high postpartum ratio suggests that protein degradation was higher after calving than before calving, although it is not known exactly when the increase in degradation began. Urine samples were not obtained at calving, so the possibility that the urinary 3-MH:creatinine ratio was highest at calving exists, and this would be consistent with the plasma 3-MH data.

Cows were at their lowest body weight at wk 4 postpartum. At that time postpartum average weight loss of cows across all treatments was 67 kg. Assuming that protein constitutes ~15% of bodyweight (Botts et al., 1979; Andrew et al., 1995) then body protein loss averaged 10 kg, consistent with Belyea et al. (1978). Motyl and Barej (1986), using urinary 3-MH output, calculated that protein degradation was 434 g/d from 5 to 10 DIM, and 380 g/d from 25 to 30 DIM. Based on these values, protein degradation during the first 28 DIM in our study would be 11.7 kg. If it is assumed that all this protein is used for milk protein synthesis (80% efficiency; Tamminga et al., 1997), then 334 g of milk protein could be produced per day, which is enough to support 10 kg of milk with 3.3% protein.

Glutamine and Gly concentrations increased at 2 d precalving, indicative of muscle protein mobilization (Verbeke et al., 1972). Skeletal muscle stores large amounts of these AA and upon degradation, releases them in substantial amounts relative to the other AA. That the majority of the AA decreased in concentration at calving is similar to Meijer et al. (1995). This decline in concentration is likely due to utilization of AA for gluconeogenesis and milk protein synthesis. The onset of lactation is associated with increased AA uptake by the mammary gland and liver (Verbeke et al., 1972). The rise in concentration by d 21 of most of the AA may be a reflection of increased protein supply, through increases in DMI and/or muscle protein degradation. Histidine and Phe were the only EAA that exhibited a decline in concentration from d 1 to d 21. Evaluation of the postpartum diet with the CNCPS (2000) model indicated that these two AA were supplied in excess of requirements, so an increase in concentration, or at least maintenance of concentration, would have been expected. Glutamine was the only

NEAA that was lower on d 21 than d 1. Based on similar observations, Meijer et al. (1995) suggested that Gln was limiting for milk protein synthesis. Considering that Gln also serves as a gluconeogenic substrate and an energy source for gut tissues, and is critical in the maintenance of immunological functions, it may be possible that it is limiting in early lactation.

2.4.3 DMI, BW, BCS, and Energy and Protein Balance

The substantial drop in intake with all treatments during the week preceding calving is consistent with previously published results (Van Saun et al., 1993; Dann et al., 1999; Greenfield et al., 2000), and may be a result of physical limitations and endocrine changes associated with parturition and lactogenesis (Grummer, 1993). Over the prepartum period, intake was similar between the HE and LE cows, while during the postpartum period, cows previously fed the HE diets consumed more feed than cows fed the LE diets, suggesting that feeding prepartum diets with a NE_L content as high as 1.65 Mcal/kg does not limit prepartum intake and may have beneficial effects on postpartum DMI.

The energy balance of cows on the HE treatments was improved throughout the prepartum and postpartum periods. Because DMI was equivalent between the HE and LE groups prepartum, the higher energy density of the HE diets increased energy intake, and hence energy balance of the HE cows was improved. Based on NRC requirements (1989), HE animals were in positive energy balance until the day of calving whereas the LE animals were in negative energy balance by the 3rd day prepartum. Consistent with the improved energy balance of the HE cows was the reduction in plasma NEFA (VandeHaar et al., 1999), the greater abundance of ACC and FAS on d 1, and the lower

hepatic TG content relative to the LE cows. Cows on the LE diets lost 0.15 BCS units between the initiation of treatment and calving, indicating that the NRC (1989) requirements may be low because the LE diets were slightly more energy dense than those recommended by NRC. VandeHaar et al. (1999) have also suggested that the NRC (1989) requirements may be too low. Indeed, NRC (2001) has raised the NE_L density required for transition cow diets to 1.62 Mcal/kg. The rationale behind this increase was the recognition of the decline in DMI of the prefresh cow, the increased energy requirements for mammary growth (although not quantified), as well as potential beneficial effects on rumen development (e.g. VFA production and papillae growth) and a possible reduction in lipid mobilization (NRC, 2001).

The higher postpartum DMI of cows on the HE treatments resulted in higher energy intakes and a less negative energy balance (Figure 2-12). However, the higher prepartum CP intake of cows on the HP treatments did not influence postpartum CP intake. Putnam and Varga (1998) also found that postpartum intake was unaffected by prepartum protein intake. However, that our cows were in negative protein balance postpartum until 42 DIM contrasts with Grummer (1995), who calculated the CP balance of cows in the study of Bertics et al. (1992) and reported that the cows had regained positive body protein balance by 7 d postpartum. This discrepancy may be due to differences in protein content of the diet, DMI, and/or milk protein yield.

2.4.4 Milk yield and composition

Prepartum dietary energy and protein levels had no significant effects on milk yield, an observation often reported by others (VandeHaar et al., 1999; Dewhurst et al., 2000; Holcomb et al., 2001). It would appear that the reliance on mobilization of body tissue

to support milk synthesis modulates the effect of prepartum treatment. Milk fat content during the first 21 DIM tended to be lower in cows previously fed HE vs. LE diets, but was not different during d 22 to d 42 (energy × time interaction, $P = 0.08$). This may reflect plasma NEFA concentrations, as Pullen et al. (1989) found a positive correlation between milk fat content and plasma NEFA. Milk FA composition did not differ among treatments but did differ with time postpartum. There was a shift from long chain to short chain FA from wk 2 to wk 6, but the relative proportion of C16 remained unchanged. This change in FA profile represents a shift between de novo FA synthesis in the mammary gland and direct uptake of NEFA from plasma. At wk 2 compared to wk 6, cows are in more negative energy balance, plasma NEFA are higher, lipogenesis is reduced, and lipolysis is enhanced. This would allow more direct incorporation of preformed long chain FA, and less de novo synthesis, as evidenced by the FA composition of milk fat at wk 2 more closely resembling that of adipose tissue (Rukkwamsuk et al., 2000) than does the composition at wk 6.

2.4.5 Nutrient Digestibility

An improvement in DM and OM digestibility, as observed with the HE diets, is commonly seen when concentrates replace forages in the diet. This effect may be attributed to an increase in starch content and a reduction in NDF (Valadares Filho et al., 2000). The improvement in CP digestibility of the HP diets compared to the LP diets is similar to that observed by Broderick et al. (2000). In that study, the inclusion of fishmeal in the diet increased N digestibility from 57.2% to 62.4%. Dilution of metabolic fecal N may account for the increase in apparent N digestibility of the HP diets (Stallcup et al., 1975). This improvement in digestibility may also be partly due to higher

ammonia production in the rumen, as evidenced by elevated PUN, with the HP diets. Broderick et al. (2000) also reported an increase in NDF digestibility with high protein diets as was observed in our study.

2.5 Conclusion

Prepartum diets containing 1.65 Mcal/kg NE_L increased postpartum DMI and improved energy balance of the cows both prepartum and postpartum, but did not result in higher milk yields compared to diets containing 1.30 Mcal/kg NE_L. The higher energy diets also reduced plasma NEFA concentrations at calving and reduced hepatic TG content. Consistent with these changes was a reduction in ACC and FAS abundance in adipose tissue of cows fed the LE diets. The CP content of the prepartum diets did not affect any of these parameters. However, the higher CP diets reduced muscle protein degradation, as evidenced by plasma 3-MH concentrations. The higher CP diets also resulted in higher PUN levels prepartum and elevated BHBA levels.

Feeding a high energy diet for 3 wk prepartum had beneficial effects on energy metabolism but these effects did not result in improvements in productivity in early lactation. Positive effects of high CP intake on productivity were negligible, although there were reductions in PUN and muscle protein degradation.

Table 2-1 Ingredient and nutrient composition of prepartum dietary treatments (DM basis).

	Treatments[1]			
	HEHP	HELP	LEHP	LELP
Ingredient composition (%):				
Alfalfa silage	6.4	6.5	6.2	6.3
Barley silage	32.9	32.4	10.5	11.0
Grass hay	17.2	13.1	31.5	30.5
Oat hulls	...	4.9	28.1	30.8
Beet pulp pellets	8.8	8.9	3.4	3.3
Rolled barley grain	19.2	28.3	6.0	11.5
Canola meal	2.5	0.4	1.3	0.4
Soybean meal	2.5	0.4	1.6	1.5
Corn gluten meal	2.5	0.4	2.8	1.0
Blood meal	3.4	...	6.1	1.0
Tallow	2.2	2.2	...	...
Vitamin ADE[2]	1.04	1.04	1.04	1.04
Magnesium oxide	0.22	0.22	0.22	0.22
Trace mineral mix[3]	0.81	0.81	0.81	0.81
Dicalcium phosphate	0.07	0.15	0.15	0.22
Limestone	0.15	0.22	0.22	0.30
Chromic oxide	0.07	0.07	0.07	0.07
Nutrient composition[4]:				
DM, %	55.2	56.2	69.5	68.9
OM, %	91.6	92.3	91.4	91.3
NE_L, Mcal/kg	1.65	1.65	1.30	1.30
CP, %	17.5	12.5	17.2	12.5
NDF, %	43.9	43.0	51.5	50.2
ADF, %	25.3	24.4	28.7	28.2
Ca, %	1.02	0.85	0.95	0.91
P, %	0.44	0.40	0.45	0.44
K, %	1.54	1.42	1.44	1.43
S, %	0.26	0.21	0.27	0.23
Cl, %	0.35	0.32	0.45	0.45
Mg, %	0.35	0.32	0.35	0.35
Na, %	0.18	0.16	0.17	0.17
Zn, ppm	137	130	110	107
Fe, ppm	373	284	421	354
Mn, ppm	136	124	157	151
Cu, ppm	47	38	37	36
DCAD, meq/kg[5]	211	211	149	165

[1]HEHP = high energy and high protein, HELP = high energy and low protein, LEHP = low energy and high protein, LELP = low energy and low protein.
[2]Supplemented the diet with 16,760 IU/kg Vitamin A, 1720 IU/kg Vitamin D, and 61 IU/kg Vitamin E.
[3]Supplemented the diet with 0.08% Ca, 0.08% P, 0.14% Na, 0.22 ppm Co, 31 ppm Cu, 1.0 ppm I, 70 ppm Mn, 0.27 ppm Se, 83 ppm Zn, and 31 ppm Fe.
[4]Calculated on analysis of individual ingredients. The energy value of grains was based on book values (NRC, 1989) and those of forages on ADF content.
[5]DCAD = dietary cation-anion difference; calculated on analysis of individual ingredients and using the equation (Na + K)- (Cl + S).

Table 2-2 Ingredient and nutrient composition of the postpartum diet (DM basis).

Ingredient composition (%):	
Alfalfa hay	10.8
Barley silage	20.8
Alfalfa silage	17.1
Rolled barley grain	25.3
Ground corn grain	7.2
Canola meal	6.3
Soybean meal	2.1
Corn gluten meal	2.3
Fish meal	2.0
Dry brewers grain	1.3
Tallow	2.0
Megalac[1]	0.5
Molasses	0.5
Mineral/vitamin premix[2]	1.8
Nutrient composition[3]:	
DM, %	57.7
OM, %	91.8
NE_L, Mcal/kg	1.73
CP, %	17.7
NDF, %	38.7
ADF, %	20.1
Ca, %	1.20
P, %	0.48
K, %	1.41
S, %	0.26
Cl, %	0.42
Mg, %	0.41
Na, %	0.33
Zn, ppm	73
Fe, ppm	240
Mn, ppm	67
Cu, ppm	21
Al, ppm	97
B, ppm	14
DCAD[4], meq/kg	221

[1] Church & Dwight Co., Inc, Princeton, NJ

[2]Supplied the diet with 0.27% Na, 0.18 ppm Co, 14 ppm Cu, 0.65 ppm I, 38 ppm Mn, 0.3 ppm Se, 40 ppm Zn, and 30 ppm Fe.

[3]Calculated on analysis of individual ingredients. The energy value of grains was based on NRC (1989) values and those of forages on ADF content.

[4]DCAD = dietary cation-anion difference; calculated on analysis of individual ingredients and using the equation (Na + K) - (Cl + S).

Table 2-3 Adipose tissue acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) abundance around the time of calving[1].

	Treatment[2]					*P*[3]				
	HEHP	HELP	LEHP	LELP	SEM	D	E	P	E × P	E × D
ACC[4]										
d –25	1.68				0.20	0.001	0.32	0.88	0.90	0.002
d 1	0.95	1.15	0.63	0.73	0.14					
d 21	0.35	0.16	0.37	0.35	0.15					
FAS[4]										
d –25	1.32				0.17	0.001	0.20	0.53	0.28	0.006
d 1	0.73	1.18	0.51	0.51	0.15					
d 21	0.06	0.05	0.22	0.08	0.16					

[1]LS means are presented with the pooled standard error of the mean (SEM).
[2]HEHP = high energy and high protein, HELP = high energy and low protein, LEHP = low energy and high protein, LELP = low energy and low protein.
[3]*P*-value for treatment and time effects: E = energy, P = protein, D = day.
[4]Data for d -25 are pooled because cows were not on treatment; data for d 1 and 21 were covariately adjusted for differences in pretreatment values; data is expressed as densitometric units/25 μg cytosolic protein

Table 2-4 Adipose tissue acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and lipoprotein lipase (LPL) activity around the time of calving[1].

	Treatment[2]					*P*[3]				
	HEHP	HELP	LEHP	LELP	SEM	D	E	P	E × P	E × D
ACC[4,5]										
d –25	10.71				1.25	0.05	0.95	0.77	0.61	0.86
d 1	1.97	1.77	1.58	2.45	0.80					
d 21	1.14	0.41	1.03	0.40	0.82					
FAS[4,6]										
d –25	265.5				53.5	0.06	0.63	0.45	0.68	0.33
d 1	262.1	213.7	337.3	220.6	53.5					
d 21	200.0	206.4	198.7	132.0	54.8					
LPL[4,7]										
d –25	498.8				138.7	0.004	0.56	0.26	0.61	0.28
d 1	247.2	453.6	427.4	537.3	110.3					
d 21	146.6	249.1	167.8	179.6	119.3					

[1]LS means are presented with the pooled standard error of the mean (SEM).
[2]HEHP = high energy and high protein, HELP = high energy and low protein, LEHP = low energy and high protein, LELP = low energy and low protein.
[3]*P*-value for treatment and time effects: E = energy, P = protein, D = day.
[4]Data for d -25 are pooled because cows were not on treatment; data for d 1 and 21 were covariately adjusted for differences in pretreatment values.
[5]Activity is expressed as nmol $H^{14}CO_3$ incorporated into malonyl-CoA/min per mg protein.
[6]Activity is expressed as nmol NADPH/min per mg protein.
[7]Activity is expressed as nmol oleic acid/h per mg protein.

Table 2-5 Plasma amino acid concentrations (μM) around the time of calving[1].

	Day						*P*[2]	
	-25[3]	-7	-2	1	21	SEM	-2 vs. 1	1 vs. 21
Essential amino acids								
Arg	77.4	81.5	77.4	57.2	68.0	3.1	0.001	0.01
His	53.5	61.4	56.8	52.4	40.9	2.3	0.15	0.001
Ile	92.5	104.3	100.7	69.8	87.1	3.7	0.001	0.001
Leu	105.9	145.0	137.7	101.3	109.4	6.7	0.001	0.39
Lys	71.6	75.6	66.1	49.7	63.4	3.3	0.001	0.001
Met	22.3	20.1	18.8	19.3	18.8	0.81	0.47	0.64
Phe	43.0	51.5	45.5	42.2	37.9	1.5	0.07	0.01
Thr	75.6	67.9	56.1	46.2	60.8	3.0	0.001	0.001
Trp	33.9	30.0	24.2	14.6	28.3	1.4	0.001	0.001
Val	187.8	228.8	208.2	140.1	160.8	10.6	0.001	0.16
Nonessential amino acids								
Ala	164.8	136.4	148.4	152.6	153.5	8.3	0.61	0.94
Asn	27.7	32.5	35.8	23.2	33.8	1.2	0.001	0.001
Asp	7.6	8.3	8.2	5.9	8.8	0.40	0.001	0.001
Cit	59.6	71.4	74.8	57.5	73.5	2.4	0.001	0.001
Gln	254.8	237.6	306.8	217.6	189.1	9.4	0.001	0.03
Glu	98.9	80.8	52.6	81.4	101.9	6.4	0.002	0.02
Gly	143.4	162.2	272.6	208.6	233.7	13.8	0.001	0.19
Orn	42.8	45.2	36.9	29.9	36.4	1.8	0.005	0.004
Ser	56.3	65.2	67.7	65.0	64.6	3.0	0.47	0.93
Tyr	47.9	50.8	37.4	34.1	42.3	1.8	0.05	0.001

[1]LS means are presented with the pooled standard error of the mean (SEM).
[2]*P*-value for day.
[3]d -25 values are pretreatment.

Table 2-6 Effect of prepartum dietary treatment on lactation performance and change in BW and BCS[1].

		Treatments[2]					P^3					
	Period	HEHP	HELP	LEHP	LELP	SEM	E	P	E × P	T	E × T	P × T
n	(wk)[4]	7	6	6	7							
DMI, kg/d	prepartum	12.8	12.3	13.9	12.8	0.9						
	1 to 3	14.4	15.2	13.3	13.0	0.9	0.18	0.66	0.79	0.001	0.01	0.33
	4 to 6	20.5	19.8	18.1	17.8	1.0						
Yield, kg/d												
Milk	1 to 3	33.8	33.4	30.9	31.4	1.9	0.22	0.62	0.55	0.001	0.83	0.12
	4 to 6	42.6	38.9	38.6	38.2	2.0						
Fat	1 to 3	1.41	1.50	1.52	1.46	0.08	0.89	0.73	0.94	0.47	0.32	0.41
	4 to 6	1.61	1.46	1.47	1.49	0.09						
Protein	1 to 3	1.11	1.10	1.05	1.07	0.07	0.55	0.65	0.55	0.02	0.65	0.18
	4 to 6	1.24	1.11	1.14	1.15	0.07						
Lactose	1 to 3	1.50	1.46	1.34	1.39	0.09	0.21	0.57	0.43	0.001	0.85	0.11
	4 to 6	1.95	1.76	1.75	1.75	0.09						
Milk composition, %:												
Fat	1 to 3	4.25	4.57	4.82	4.78	0.21	0.28	0.81	0.60	0.001	0.08	0.36
	4 to 6	3.82	3.81	3.89	3.82	0.22						
Protein	1 to 3	3.29	3.41	3.26	3.41	0.09	0.80	0.30	0.69	0.001	0.53	0.36
	4 to 6	2.90	2.88	2.90	2.98	0.09						
Lactose	1 to 3	4.41	4.40	4.36	4.42	0.06	0.93	0.88	0.58	0.001	0.75	0.31
	4 to 6	4.56	4.52	4.53	4.54	0.06						
BCS, prepartum[5]		3.01	3.17	3.19	3.11	0.09	0.21	0.94	0.51	0.001	0.13	0.38
postpartum[6]		2.67	2.64	2.82	2.81	0.09						
BCS change[7]		-0.02	-0.02	-0.19	-0.11	0.09	0.19	0.65	0.65			
BCS change[8]		-0.55	-0.65	-0.43	-0.54	0.09	0.23	0.29	0.98			
BW change, kg[7]		35.6	24.0	37.5	23.7	9.3	0.93	0.19	0.91			
BW change, kg[8]		-57.9	-46.3	-59.9	-56.4	9.6	0.53	0.47	0.68			

[1]LS means are presented with the pooled standard error of the mean (SEM).
[2]HEHP = high energy and high protein, HELP = high energy and low protein, LEHP = low energy and high protein, LELP = low energy and low protein.
[3]The statistical model contained pre- and postpartum data, and included period as a repeated measure; *P*-value for treatment and time effects: E = energy, P = protein, T = period
[4]Refers to weeks postpartum.
[5]Mean BCS from 3 wk precalving to calving.
[6]Mean BCS from calving to 6 wk postcalving.
[7]Difference between pretreatment data and calving data.
[8]Difference between calving data and data at 6 wk postcalving.

Table 2-7 Milk fatty acid composition at 2 and 6 wk postpartum[1].

	Weeks postpartum			
	2	6	SEM	P^2
Fatty acid, %				
C4:0	2.09	1.96	0.05	0.10
C6:0	2.93	3.68	0.10	0.001
C8:0	0.93	1.39	0.05	0.001
C10:0	1.70	2.85	0.12	0.001
C12:0	1.90	3.24	0.12	0.001
C14:0	7.25	10.25	0.29	0.001
C14:1	0.69	0.93	0.04	0.001
C15:0	0.82	1.06	0.03	0.001
C16:0	27.64	27.59	0.20	0.86
C16:1	2.69	2.24	0.07	0.001
C18:0	12.06	10.25	0.28	0.001
C18:1	29.67	24.35	0.58	0.001
C18:2	2.26	2.02	0.06	0.009
C18:3	0.44	0.41	0.01	0.12
C20:0	0.19	0.21	0.01	0.13
C20:1	0.39	0.46	0.02	0.006
C20:4	0.14	0.12	0.005	0.001
C20:5	0.04	0.07	0.007	0.004
C22:0	0.09	0.11	0.008	0.05
C22:1	0.09	0.07	0.03	0.52
Short chain[3]	18.32	25.37	0.70	0.001
Medium chain[4]	30.33	29.83	0.22	0.10
Long chain[5]	46.19	38.76	0.77	0.001

[1]LS means are presented with the pooled standard error of the mean (SEM).
[2]*P*-value for week.
[3]Includes C4:0 to C15:0.
[4]Includes C16:0 and C16:1.
[5]Includes C18:0 to C22:1.

Table 2-8 Digestibility of nutrients in cows fed different diets prepartum[1].

	Treatments[2]					*P*[3]		
	HEHP	HELP	LEHP	LELP	SEM	E	P	E × P
Prepartum digestibility, %								
DM	71.4	67.2	64.0	64.0	1.4	0.002	0.15	0.18
OM	74.7	70.5	66.5	67.2	1.4	0.001	0.23	0.11
CP	71.1	61.0	68.0	61.4	2.0	0.52	0.001	0.42
ADF	54.7	45.1	49.7	51.3	2.6	0.81	0.15	0.05
NDF	63.7	55.1	58.5	55.9	2.3	0.33	0.03	0.22
Postpartum digestibility, %								
DM	73.8	76.1	74.1	74.4	2.0	0.74	0.52	0.66
OM	76.1	77.8	75.9	76.8	1.8	0.75	0.49	0.83
CP	74.3	76.9	74.9	75.4	1.9	0.84	0.43	0.61
ADF	55.4	51.5	48.2	49.8	2.3	0.09	0.62	0.26
NDF	64.4	61.0	59.7	63.1	1.8	0.49	0.99	0.10

[1]LS means are presented with the pooled standard error of the mean (SEM).
[2]HEHP = high energy and high protein, HELP = high energy and low protein, LEHP = low energy and high protein, LELP = low energy and low protein.
[3]*P* -value for treatment effect: E = energy, P = protein.

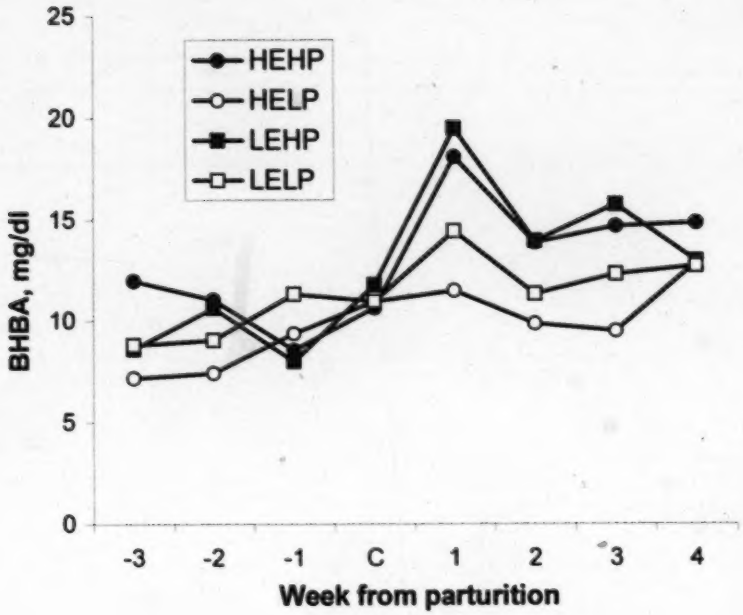


Figure 2-1 Plasma ß-hydroxybutyrate (BHBA) concentrations around the time of calving (pooled SEM = 1.99). Treatment effects: protein, $P = 0.03$. Cows were fed diets for 21 d before calving that contained a high (HE) or low (LE) concentration of energy and a high (HP) or low (LP) concentration of protein. All cows were fed the same diet postcalving. The data for calving (C) were obtained within 24 h of calving.

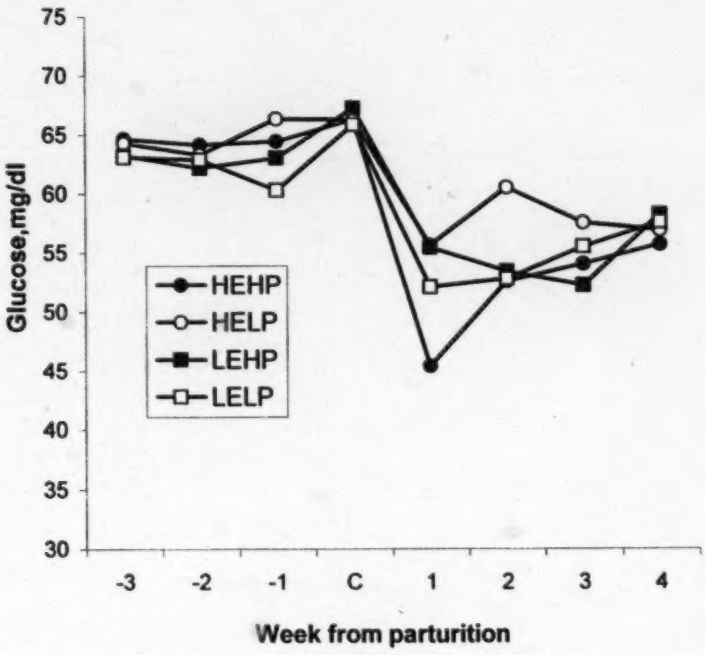


Figure 2-2 Plasma glucose concentrations around the time of calving (pooled SEM = 2.75). There were no treatment effects ($P > 0.05$). Cows were fed diets for 21 d before calving that contained a high (HE) or low (LE) concentration of energy and a high (HP) or low (LP) concentration of protein. All cows were fed the same diet postcalving. The data for calving (C) were obtained within 24 h of calving.

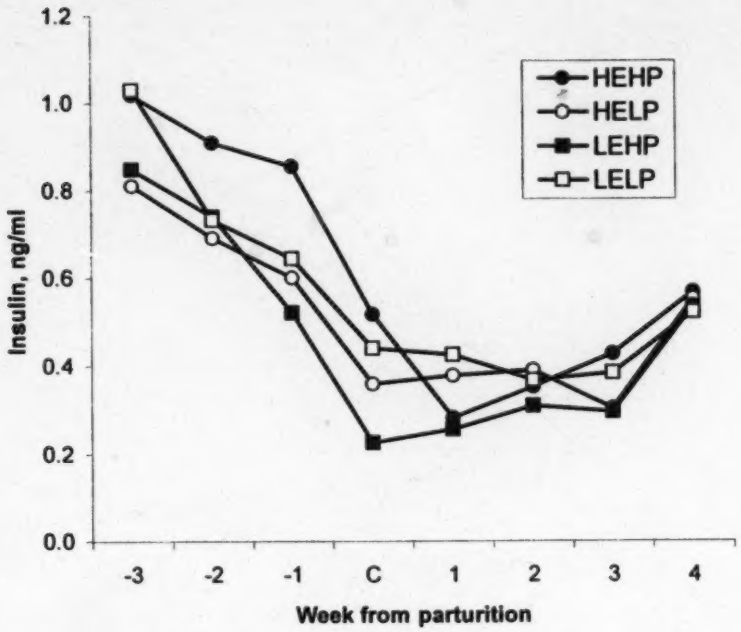


Figure 2-3 Plasma insulin concentrations around the time of calving (pooled SEM = 0.09). Treatment effect: energy × protein, $P = 0.06$. Cows were fed diets for 21 d before calving that contained a high (HE) or low (LE) concentration of energy and a high (HP) or low (LP) concentration of protein. All cows were fed the same diet postcalving. Data were covariately adjusted for differences in pretreatment values. The data for calving (C) were obtained within 24 h of calving.

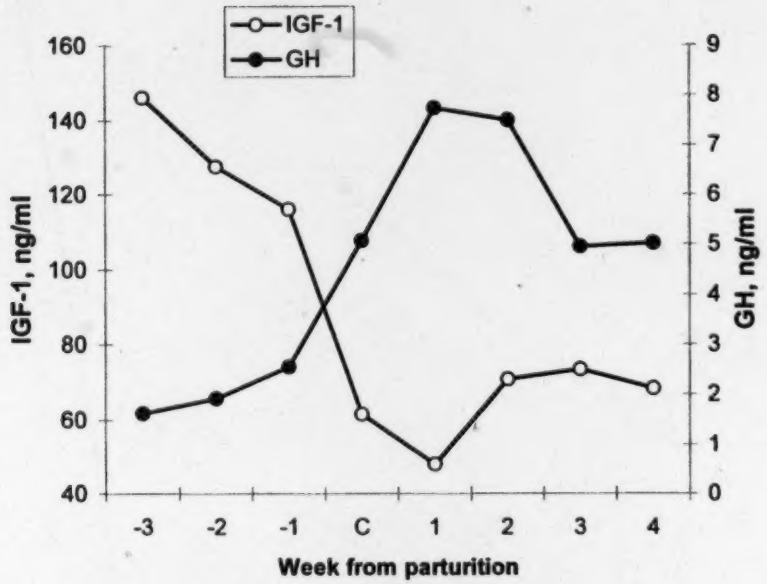


Figure 2-4 Plasma IGF-1 (pooled SEM = 8.60) and growth hormone (GH) concentrations (pooled SEM = 1.23) around the time of calving. There were no treatment effects ($P > 0.10$). Cows were fed diets for 21 d before calving that contained a high (HE) or low (LE) concentration of energy and a high (HP) or low (LP) concentration of protein. All cows were fed the same diet postcalving. Data were covariately adjusted for differences in pretreatment values. The data for calving (C) were obtained within 24 h of calving.

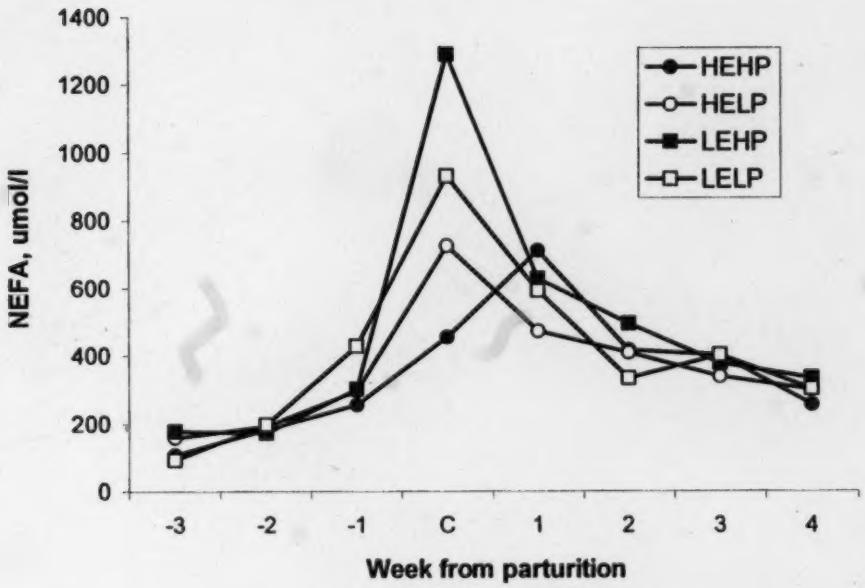


Figure 2-5 Plasma nonesterified fatty acid (NEFA) concentrations around the time of calving (pooled SEM = 93.7). Treatment effects: energy × week, $P < 0.001$; energy × protein × week, $P = 0.005$. Cows were fed diets for 21 d before calving that contained a high (HE) or low (LE) concentration of energy and a high (HP) or low (LP) concentration of protein. All cows were fed the same diet postcalving. The data for calving (C) were obtained within 24 h of calving.

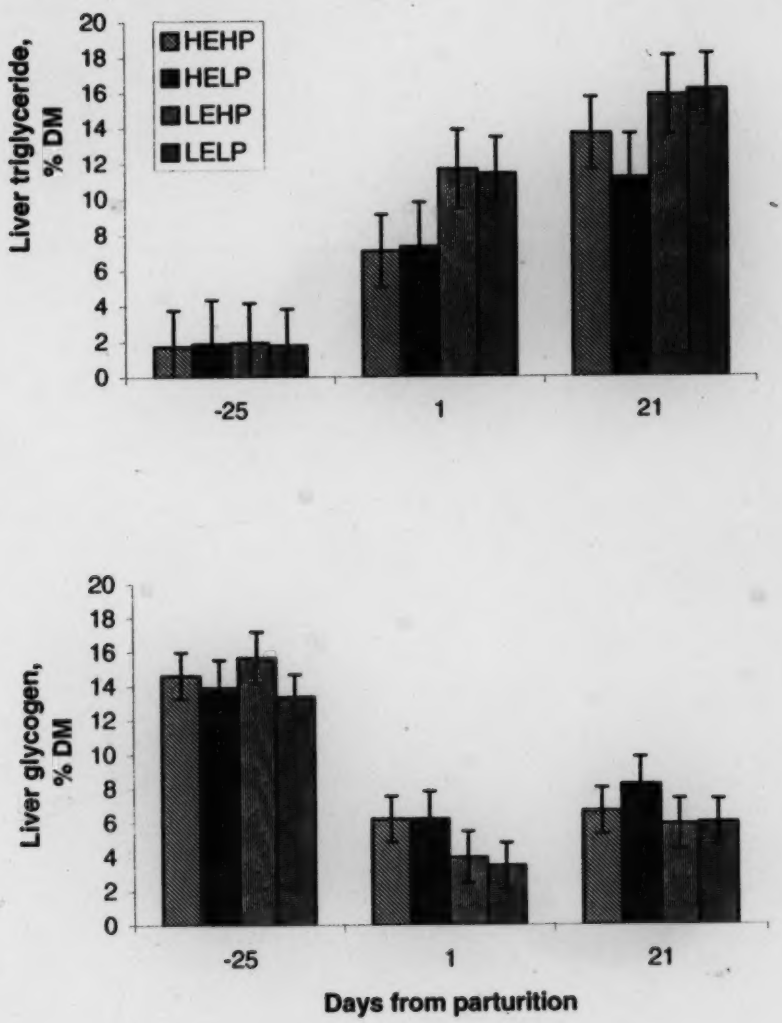


Figure 2-6 Concentrations of liver triglyceride (upper panel; pooled SEM = 2.17) and glycogen (lower panel; pooled SEM = 1.44) (DM basis) of cows during the transition period. Treatment effects for liver triglyceride: energy, $P = 0.06$. There were no treatment effects on liver glycogen ($P > 0.05$). Cows were fed diets for 21 d before calving that contained a high (HE) or low (LE) concentration of energy and a high (HP) or low (LP) concentration of protein. All cows were fed the same diet postcalving. Day -25 data are pretreatment.

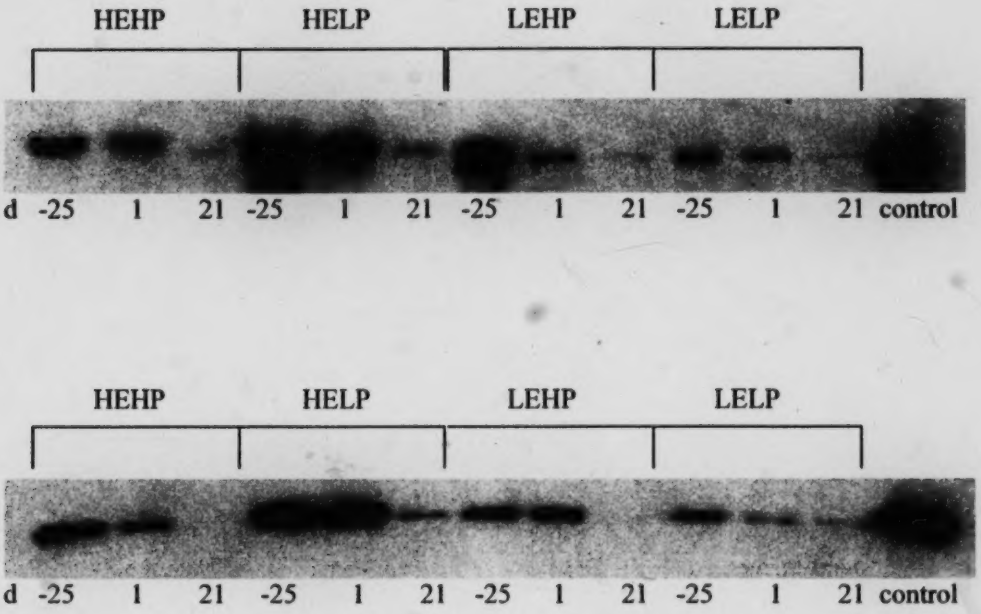


Figure 2-7 Western blot of ACC protein (265 kDa, upper panel) and FAS protein (260 kDa, lower panel) in adipose tissue. Each treatment within a block was represented on the same gel. Data from one cow is represented for each treatment. Cows were fed diets for 21 d before calving that contained a high (HE) or low (LE) concentration of energy and a high (HP) or low (LP) concentration of protein. All cows were fed the same diet postcalving.

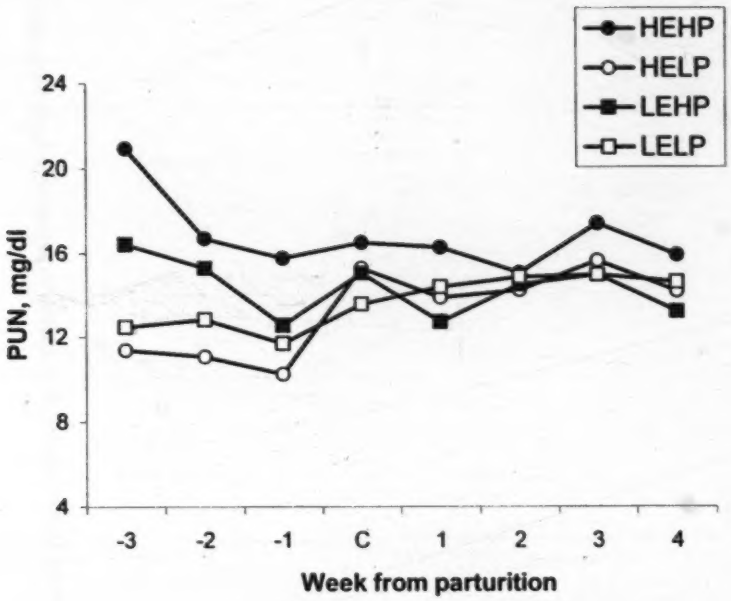


Figure 2-8 Plasma urea nitrogen (PUN) concentrations around the time of calving (pooled SEM = 1.18). Treatment effects: protein × energy, $P = 0.05$; protein × week, $P = 0.003$. Cows were fed diets for 21 d before calving that contained a high (HE) or low (LE) concentration of energy and a high (HP) or low (LP) concentration of protein. All cows were fed the same diet postcalving. The data for calving (C) were obtained within 24 h of calving.

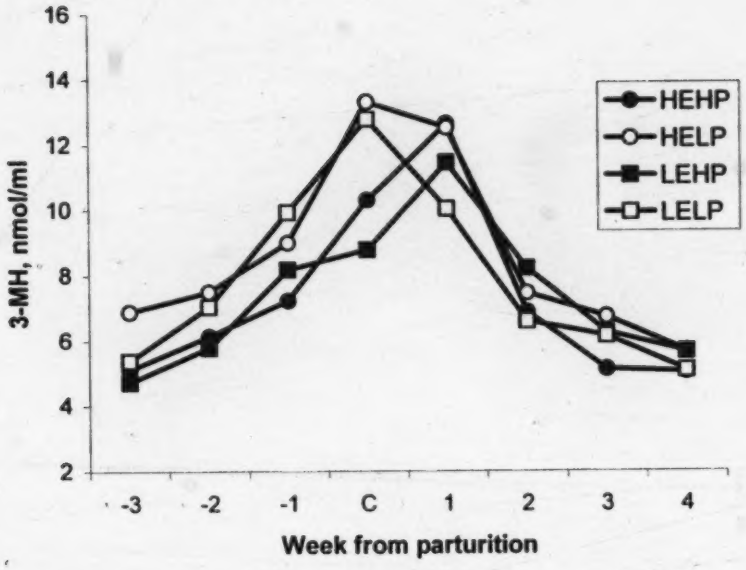


Figure 2-9 Plasma 3-methylhistidine (3-MH) concentrations around the time of calving (pooled SEM = 1.18). Treatment effect: protein × week, $P = 0.004$. Cows were fed diets for 21 d before calving that contained a high (HE) or low (LE) concentration of energy and a high (HP) or low (LP) concentration of protein. All cows were fed the same diet postcalving. The data for calving (C) were obtained within 24 h of calving.

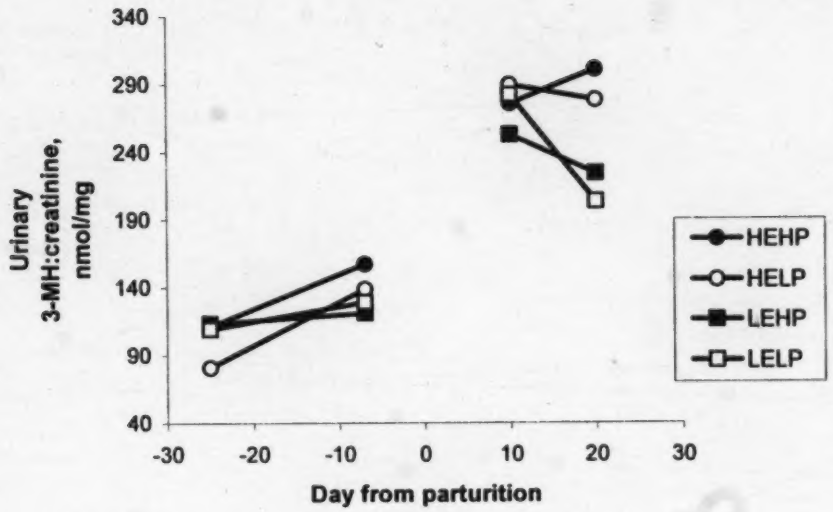


Figure 2-10 Urinary 3-methylhistidine (3-MH):creatinine ratio (pooled SEM = 26.8) around the time of calving. Treatment effect: energy × day, $P = 0.1$. Cows were fed diets for 21 d before calving that contained a high (HE) or low (LE) concentration of energy and a high (HP) or low (LP) concentration of protein. All cows were fed the same diet postcalving. Day -25 data are pretreatment.

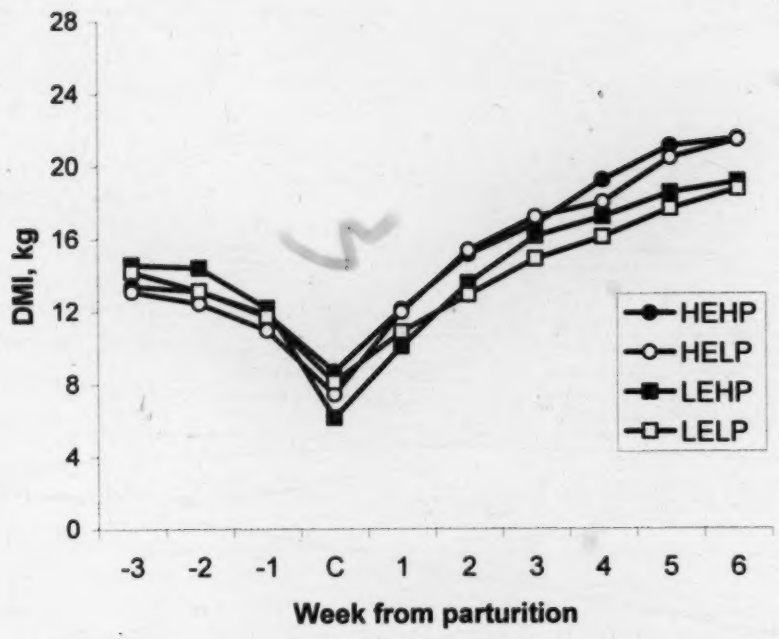
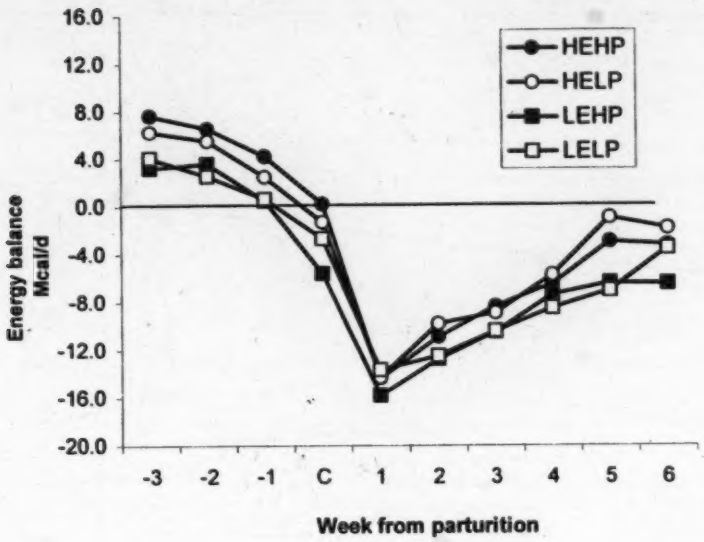


Figure 2-11 Daily DMI during the periparturient period (pooled SEM = 1.04). Treatment effects: energy by period, $P < 0.01$. Cows were fed diets for 21 d before calving that contained a high (HE) or low (LE) concentration of energy and a high (HP) or low (LP) concentration of protein. All cows were fed the same diet postcalving. The data for wk represents the average of 7 d while the data for calving (C) was obtained from the day of calving only.



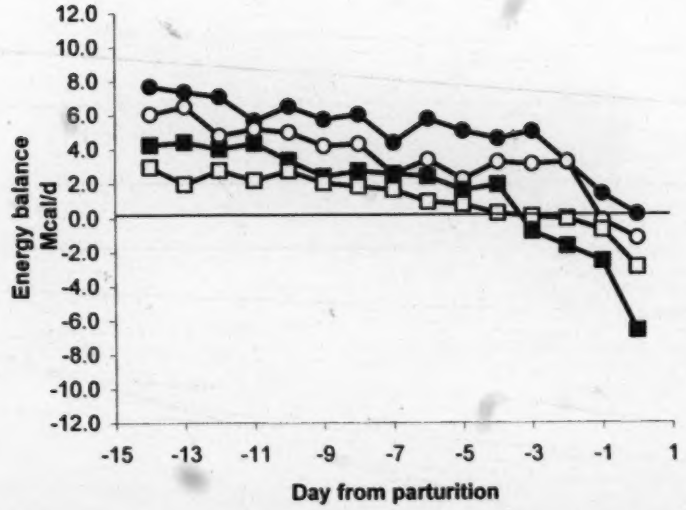
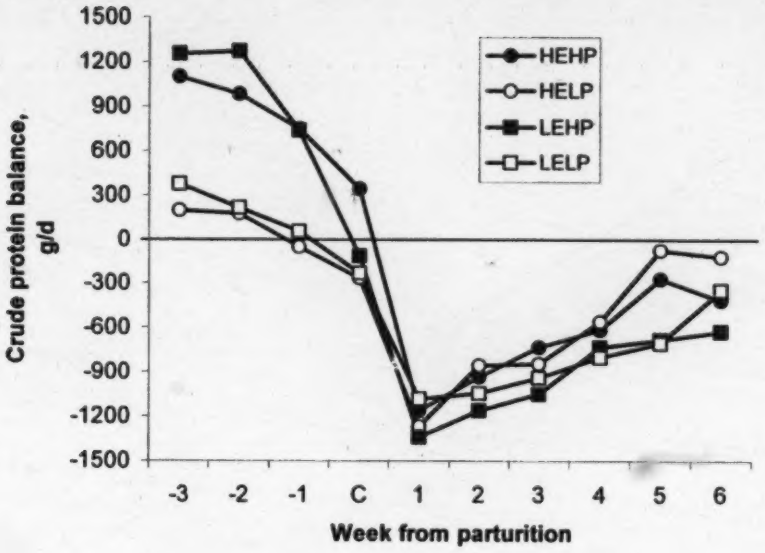


Figure 2-12 Net energy balance during the periparturient period (pooled SEM = 1.61). Treatment effects: energy, $P = 0.003$. Cows were fed diets for 21 d before calving that contained a high (HE) or low (LE) concentration of energy and a high (HP) or low (LP) concentration of protein. All cows were fed the same diet postcalving. The data for wk represents the average of 7 d while the data for calving (C) was obtained from the day of calving only.



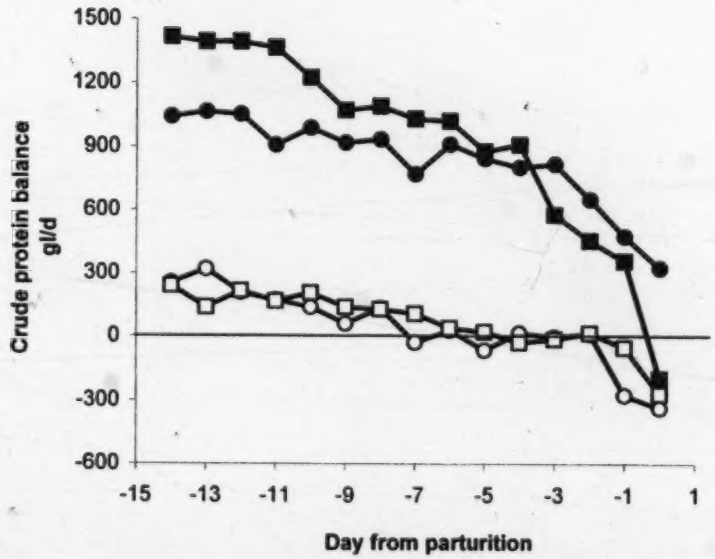


Figure 2-13 Crude protein balance during the periparturient period (pooled SEM = 169). Treatment effects: protein, $P = 0.006$; protein × week, $P < 0.0001$. Cows were fed diets for 21 d before calving that contained a high (HE) or low (LE) concentration of energy and a high (HP) or low (LP) concentration of protein. All cows were fed the same diet postcalving. The data for wk represents the average of 7 d while the data for calving (C) was obtained from the day of calving only.

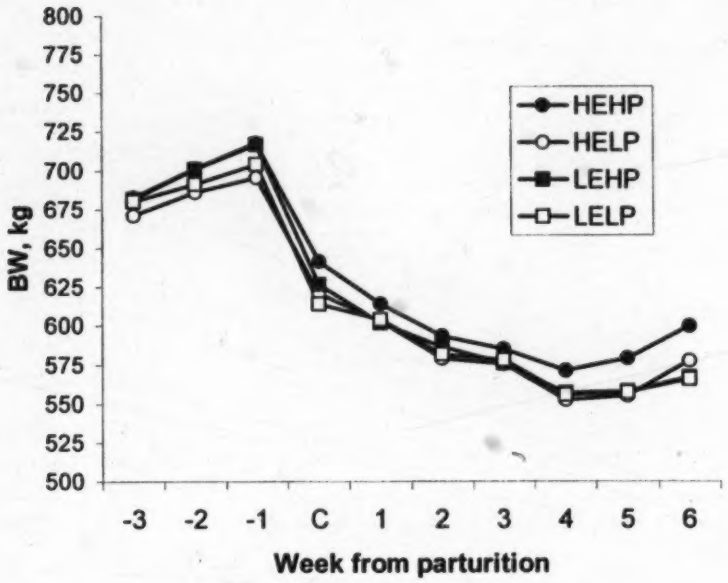


Figure 2-14 Bodyweight (BW) around the time of calving (pooled SEM = 11.1). There were no treatment effects ($P > 0.05$). Cows were fed diets for 21 d before calving that contained a high (HE) or low (LE) concentration of energy and a high (HP) or low (LP) concentration of protein. All cows were fed the same diet postcalving. Data were covariately adjusted for differences in pretreatment values. The data for calving (C) were obtained within 24 h of calving.

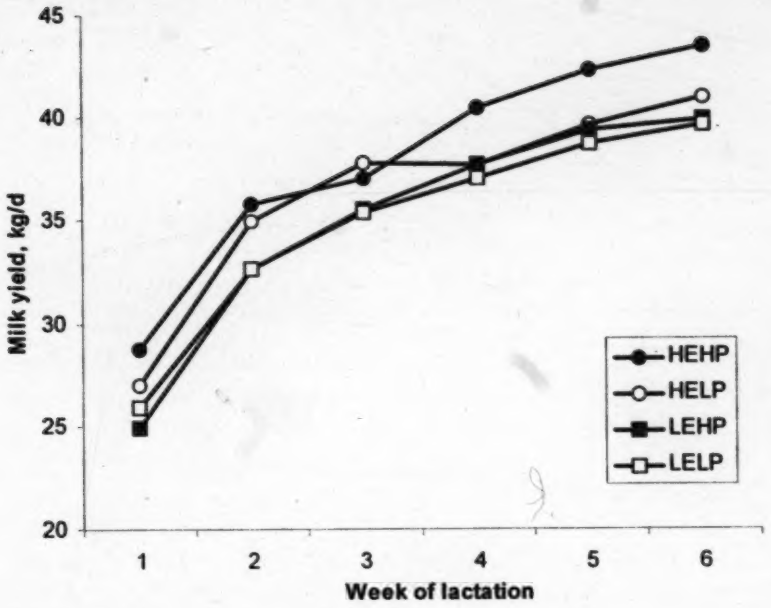


Figure 2-15 Milk yield during the first 6 wk postpartum (pooled SEM = 2.1). There were no treatment effects ($P > 0.05$). Cows were fed diets for 21 d before calving that contained a high (HE) or low (LE) concentration of energy and a high (HP) or low (LP) concentration of protein. All cows were fed the same diet postcalving.

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CHAPTER 3

Plasma Amino Acid Concentrations During the Periparturient Period

3.1 Introduction

During the periparturient period the nutrient demands of dairy cows frequently exceed nutrient intake. Precalving, this is a result of increased demands for fetal growth and mammary gland development in conjunction with a decline in feed intake. Postcalving, the nutrient deficit results from demands for milk synthesis accompanied by insufficient feed intake. Mobilization of body lipid supplies the majority of the deficient energy substrates although mobilization of body proteins also contributes to making up the deficit. Komaragiri and Erdman (1997) reported that cows mobilized 21 kg of body protein from 14 d prepartum to 35 d postpartum. The AA that are released upon protein mobilization are used during the prepartum period primarily for fetal protein synthesis and as energy substrates, and during the postpartum period, for synthesis of milk protein as well as hepatic gluconeogenesis (Bell, 1995). The equilibrium between AA supply and demand is partly reflected in the plasma AA concentrations. As described in chapter 1, plasma AA concentrations have been used as indices of AA limitation, although there are restrictions to the use of this technique. Regardless, a decline in one AA relative to other AA in plasma may indicate that this AA is limiting production. For example, Meijer et al. (1995) suggested that Gln was limiting for milk protein synthesis based on its plasma concentration remaining depressed for the first 15 wk of lactation while the remainder of the AA increased in concentration.

The few studies in which plasma AA concentrations during the transition period have been reported have demonstrated pronounced changes around parturition (Meijer et

al., 1995; Motyl and Barej, 1986; Verbeke et al., 1972; chapter 2 of this thesis). This experiment was conducted to determine if changes in plasma AA concentration, relative to one another, could indicate specific AA limitations. Secondly, by comparing results to previous studies, the objective was to determine if peripartum changes in AA concentrations are influenced primarily by the diet of the cow or its physiological state.

3.2 Materials and Methods

3.2.1 Diets and sampling

The animals used in this study were part of a larger study conducted at the Dairy and Swine Research and Development Centre, Lennoxville, Quebec. In brief, 24 multiparous Holstein cows were assigned to one of six blocks on the basis of parity and milk production during their previous lactation. Within each block, animals were randomly assigned to one of four treatments – control, vitamin B12 at 500 mg/d, folic acid at 2.5 g/d, and vitamin B12 and folic acid at 500 mg/d and 2.5 g/d, respectively. Treatments were initiated 3 wk before expected calving date and terminated 8 wk postcalving. The vitamin supplements were administered once daily by mixing them with a small amount of concentrate and top-dressing this on a small amount of TMR. Cows were fed the balance of the TMR after the vitamin supplements were completely ingested. Cows were fed once daily precalving and twice daily postcalving. In both periods, cows were fed a TMR ad libitum. Ingredient composition and formulated nutrient composition of the TMRs are in Table 3-1. Forage NE_L content was calculated from Minnesota Valley Testing Laboratories: [1.044-(0.0123 × ADF)] × 2.2046 for legumes, [1.085-(0.015 × ADF)] × 2.2046 for grasses, and [0.94-(0.008 × ADF)] × 2.2046 for corn silage, where NE_L density is in Mcal/kg, and ADF is in percentage of

DM. To be consistent with calculations in chapter 2, the NE_L content of the concentrates was based on NRC (1989) values. In addition, cows were fed 3.5 kg (2.9 kg DM) grass hay in the precalving period, and 2.0 kg (1.7 kg DM) in the post-calving period (Table 3-1). In order to make ration adjustments and to determine DMI, moisture content of the silages was determined weekly. Based on 12 kg/d DMI prepartum and 20 kg/d postpartum, metabolizable protein supply was calculated (NRC, 2001) to be 1160 g/d and 2269 g/d, respectively.

Cows were milked twice daily at 12 h intervals. Cows were weighed and body condition scored weekly throughout the trial. Blood samples were collected from the tail vein into evacuated tubes containing EDTA on d -22 (pre-treatment), -3, 1, 14, 28, 42, and 56 relative to calving. Tubes were immediately placed on ice and centrifuged at 3000 x g for 15 min within 4 h. Plasma amino acids were quantified by HPLC using the procedure of Sedgwick et al. (1991). The procedure is described in Appendix A.

3.2.2 Statistical analysis

Repeated measures data were analyzed using the MIXED procedure of SAS (1999). The statistical model included block, treatment, time, and the treatment by time interaction. For each analyzed variable with equal time spacing between samples, cow nested within treatment by block was subjected to three covariance structures: autoregressive order 1, compound symmetry, and spatial power law. For variables with unequal spacing (plasma AA concentrations) only compound symmetry and spatial power law covariance structures were used. The covariance structure that resulted in the largest Schwarz's Bayesian Criterion and Akaike's Information Criterion was used. Preplanned comparisons for the effect of time were analyzed by PDIFF.

3.3 Results

Because of variation in gestation length, some cows were on treatment for less than the targeted 21 d precalving, therefore, prepartum DMI is reported for the last 2 wk precalving. Dry matter intake throughout the trial, i.e., 2 wk before calving to 8 wk after calving was unaffected by treatment ($P > 0.10$; Figure 3-1) and averaged 12.2 kg (SEM = 0.46) prepartum and 19.4 kg (SEM = 0.66) postpartum. The reduction in DMI from d –7 to the day of calving averaged 18.1%. No differences between treatments were observed for BW (Figure 3-2) or BCS. Body condition score averaged 3.49 (SEM = 0.07) in the prepartum period and 2.88 (SEM = 0.07) in the postpartum period. The loss in BCS during the first 8 wk of lactation was 0.52 units (SEM = 0.16), and the loss in BW was 29.7 kg (SEM = 10.63). Treatment had no effect on milk yield ($P > 0.10$), which averaged 38.5 ± 0.92 kg/d (Figure 3-3), milk protein content (3.06 ± 0.03%) or milk fat content (3.90 ± 0.08%).

Plasma AA concentrations are shown in Table 3-2. Data are pooled and presented by day of sampling only because the majority of the AA were unaffected by treatment. The exceptions to this were Ala and Ser. There was a day by treatment effect ($P = 0.04$) for Ala. On d –22 there were no differences between the four treatments but during the postpartum period, Ala was higher on the vitamin B12-folic acid treatment than on the other treatments. There was a tendency for Ser to be higher in cows fed the vitamin B12-folic acid treatment than for cows fed the other treatments ($P = 0.06$).

All the EAA concentrations decreased at calving relative to d –22 ($P \leq 0.05$). With the exception of His and Met, EAA concentrations returned to, or exceeded, prepartum (d –22) levels by d 14 postpartum. Day 14 concentrations were higher than prepartum

concentrations for Arg, Ile, Leu, Thr, Trp, and Val, and remained higher for the duration of the postpartum period. On d 14, concentrations of Lys and Phe were the same as d –22 concentrations, but by 42 d postpartum, they exceeded prepartum concentrations. Histidine and Met concentrations returned to prepartum levels by 28 d postpartum. At no point did postpartum concentrations of these AA exceed their concentrations on d –22.

The NEAA did not show the same consistent decline in concentration at calving as the EAA. Only Ala, Asn, Asp, Glu, Orn, and Tyr plasma concentrations decreased at calving ($P < 0.1$) relative to d -22, while the concentrations of Cit, Gln, and Ser were unchanged, and those of Gly increased. Plasma concentrations either remained the same or increased from d -22 to d 14 for the majority of the NEAA; the exceptions were Glu, Gln, and Orn, whose concentrations decreased. With the exception of Glu and Gln, NEAA concentrations from d 28 to d 56 were equal to, or higher, than prepartum concentrations.

3.4 Discussion

Prepartum DMI was similar to previously published values (Minor et al., 1998; VandeHaar et al., 1999). The decline in intake of 18% in the immediate prepartum period was similar to published results, being higher than some reports (<10% - Grum et al., 1996; 0% - Huyler et al., 1999) but lower than others (Chapter 2; 30% - Dann et al., 1999; 30% - Greenfield et al., 2000; 36% - VandeHaar et al., 1999). The reduction in BW and BCS during the periparturient period is consistent with Dewhurst et al. (2000), Dann et al. (1999), and Putnam and Varga (1998).

The effect of the vitamin treatments on the plasma AA concentrations is beyond the scope of this thesis, therefore, discussion is limited to the effect of physiological state,

i.e., gestation vs. lactation. Calving and the onset of lactation were associated with a transient decrease in plasma EAA concentrations, likely resulting from the large and rapidly increasing demands for AA of the mammary gland, both for milk protein synthesis and as energy precursors, as well as the increased demand of the liver for gluconeogenic precursors (Verbeke et al., 1972; Meijer et al., 1995). Contributing to the decline in plasma AA concentrations is the low DMI that occurs around parturition. Essential AA concentrations (except for Trp) did not decline between d –22 and d –3, suggesting that the amount of absorbable EAA derived from rumen microbial protein, RUP, and body mobilization was sufficient to meet demands during this period. Plasma 3-MH data from chapter 2 of this thesis suggests that protein mobilization begins 1 to 2 wk prepartum, thus contributing to the maintenance of plasma EAA concentrations. The fact that plasma AA concentrations could not be maintained at calving suggests that mobilization of body protein is unable to keep pace with demand.

By d 14 postpartum, concentrations of EAA except His and Met, and of NEAA except Glu, Gln, and Orn, returned to, or exceeded their concentrations at d –22. This likely does not reflect dietary adequacy, but rather mobilization of body protein, which can be quite substantial in early lactation. In chapter 2, the pattern of change in plasma 3-MH concentrations suggests that postpartum mobilization of body protein is high for the first wk of lactation. Bell et al. (2000) suggested that during the first 7 to 10 d of lactation cows may mobilize as much as 1000 g/d of body protein.

The return to prepartum EAA concentrations by d 14 postpartum in this study was not observed in the study described in chapter 2. In that study, plasma concentrations on d 21 were less than those on d –25 for all EAA except Ile and Leu. This difference

between the two studies may be explained by the greater feed intake of the cows in the current study compared to the cows described in chapter 2, but similar amounts of body tissue mobilization. Dry matter intake of cows in the current study for the first 3 wk of lactation was 2.21%, 2.70%, and 2.92% of BW while for the cows in the previous study, DMI was only 1.80%, 2.37%, and 2.78% of BW. Because of the higher DMI, MP supply, as calculated by CNCPS (2000), of the cows in this study accounted for 74% of the protein found in milk, whereas for the cows in chapter 2, it only accounted for 66%. Body tissue mobilization, as measured by the change in BCS was equivalent between the two sets of cows, as cows in the current study lost 0.5 BCS units between wk –1 and wk 3 and cows in chapter 2 lost 0.4 BCS units.

Like the EAA, the concentrations of several NEAA (Ala, Asn, Asp, Glu, Orn, Tyr) were lower at calving relative to d -22. This may indicate that body protein mobilization and/or the synthesis of NEAA may not occur at a fast enough rate to meet the needs of calving and galactopoiesis. Concentrations of Cit, Gln, and Ser were unchanged while that of Gly increased. The increase in the Gly plasma concentration was also observed in the study described in chapter 2, and is indicative of protein mobilization (Meijer et al., 1995). In the rat, Gly comprises 4.0% of muscle protein, but 10% of the AA released from the hindquarter, third only behind Ala and Gln (Ruderman and Berger, 1974). Also contributing to the rise in the plasma Gly concentration is the low demand for this AA for milk protein synthesis.

As in chapter 2 of this thesis, Gln decreased in concentration in the postpartum period and did not recover even by 56 DIM. This observation is consistent with Zhu et al. (2000), and Meijer et al. (1995). In the study of Meijer et al. (1995), postcalving

plasma Gln concentrations fell by 25% and muscle concentrations decreased by 30%. This marked reduction persisted until 15 wk postpartum. The reduction in concentration is the result of the widespread demands on Gln. It serves as a fuel for the gastrointestinal tract, and with the increases in DMI in early lactation and the concomitant hypertrophy of the gut, its requirement would be increased. Additionally, hepatic usage of Gln may be increased because of increased gluconeogenesis in early lactation (Heitmann et al., 1973). As well, demand by the mammary gland may be increased in early lactation (Meijer et al., 1995). Glutamate concentrations followed a similar pattern to that of Gln. These observations led Meijer et al. (1995) to suggest that Gln and Glu may be limiting AA for high yielding cows in early lactation. Further research is required to clarify glutamine's, and possibly glutamate's role as quasi-essential AA.

An interesting observation in this study is that plasma AA concentrations at d 56, except those of His, Met, Asp, and Orn, which were unchanged, and Gln and Glu, which were lower, were considerably higher than at d –22. It is possible that the elevated concentrations resulted from a demand for Gln. When a single nutrient is deficient, tissues stores are depleted so that normal concentrations can be maintained in the bloodstream (Lacey and Wilmore, 1990). If Gln is deficient, as evidenced by its low concentration throughout the postpartum period relative to its prepartum concentration, then body tissue reserves may be mobilized to meet its needs. As a result, the supply of the other AA may be greater than their demand, and the surplus will accumulate in the bloodstream. Interestingly, this did not occur for His and Met, suggesting that these two AA are also potentially limiting for milk production in early lactation.

3.5 Conclusion

Vitamin B12 and folic acid supplementation in the prepartum and early postpartum period had no effect on cow productivity, including DMI and milk production. Dry matter intake showed the typical decline for multiparity cows in the immediate prepartum period and at calving. The majority of the plasma AA concentrations were not influenced by the vitamin treatments, but did vary with time relative to calving. From 3 wk precalving to 1 d postcalving, the concentration of all the EAA decreased, indicative of increased AA demands for lactogenesis. With the exception of His and Met, EAA concentrations returned to, or exceeded, prepartum (d -22) levels by 14 d postpartum, likely a result of increasing AA intake and/or mobilization of body protein reserves. The majority of the NEAA also declined in concentration from 3 wk prepartum to 1 d postpartum, the exceptions being Cit, Gln, Gly, and Ser. With the exception of Gln, Glu, and Orn, a return to prepartum concentrations was seen by 14 d postpartum. The continued depression of Gln and Glu until 8 wk postpartum suggests that these AA may be limiting in early lactation. Further research is required to test this hypothesis.

Table 3-1 Ingredient and nutrient composition of the TMR and hay (DM basis).

	TMR		Grass Hay	
	Precalving	Postcalving	Precalving	Postcalving
Ingredient composition (%):				
Alfalfa silage	32.2	29.1		
Corn silage	27.3	19.8		
Corn grain	29.6	35.2		
Soybean meal	7.1	9.0		
Protein supplement[1]	1.9	4.1		
Mineral/vitamin mix	1.9	2.5		
Limestone	-----	0.3		
Nutrient composition (%):				
NE_L, Mcal/kg	1.59	1.62	0.97	1.21
CP, %	16.7	18.1	8.3	14.7
NDF, %	32.9	29.4	73.0	63.0
ADF, %	21.3	18.7	43.0	35.8

[1]Consisted of 30% soybean meal, 25% corn gluten meal, 20% dried wheat distillers grains, and 25% Soyplus (West Central Soy, Ralston, IA).

Table 3-2 Plasma amino acid concentrations (μM) around the time of calving[1].

	Day[2]							
	-22	-3	1	14	28	42	56	SEM
Essential amino acids								
Arginine	55.9	65.9*	43.4**	63.5†	67.9**	79.7**	78.1**	3.52
Histidine	59.3	52.7	46.3**	49.0*	52.4	57.0	56.8	3.81
Isoleucine	89.7	94.0	60.6**	114.5**	103.9†	122.0**	112.8**	5.52
Leucine	110.3	119.3	88.5*	161.1**	151.3**	180.1**	171.3**	7.20
Lysine	60.4	54.5	39.1**	59.6	57.7	74.1**	71.9*	3.61
Methionine	20.8	19.2	17.8*	18.5†	18.8	21.9	21.6	0.93
Phenylalanine	44.1	41.4	36.4**	45.0	43.7	51.0**	48.2†	1.83
Threonine	63.7	60.7	47.7*	82.1*	90.0**	113.8**	97.1**	5.96
Tryptophan	34.6	24.1**	17.7**	40.6*	38.5	42.4**	45.7**	2.09
Valine	169.3	176.5	118.6**	222.8**	219.6**	253.2**	244.9**	9.70
Nonessential amino acids								
Alanine	192.6	152.7**	141.8**	183.5	183.6	234.8**	228.8**	9.96
Asparagine	28.1	27.4	22.2†	42.7**	43.7**	52.7**	49.0**	2.20
Aspartate	7.8	7.7	6.4*	7.9	7.8	9.1	8.6	0.56
Citrulline	56.2	71.1*	53.4	85.3**	90.7**	80.7**	85.7**	4.85
Glutamine	260.0	274.7	260.7	221.3**	217.4**	239.8	225.5*	11.03
Glutamate	63.5	65.5	55.5*	47.6**	47.1**	50.5**	43.2**	2.59
Glycine	170.4	210.0	233.4*	448.1**	358.3**	312.2**	284.9**	19.9
Ornithine	35.0	33.9	23.6**	30.1†	31.7	42.4**	39.1	2.03
Serine	58.6	72.7†	69.0	90.9**	83.1**	95.2**	81.4**	5.18
Tyrosine	46.1	45.4	36.3*	53.6†	55.9*	65.4**	64.4**	3.03

[1] LS means are presented with the pooled standard error of the mean (SEM).

[2] d –22 values are pre-treatment.

† $P < 0.10$; * $P < 0.05$; ** $P < 0.01$; all comparisons are made relative to d –22.

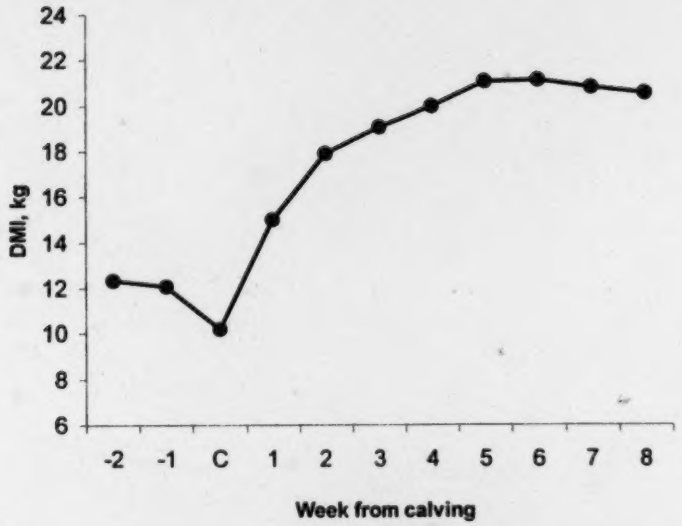

Figure 3-1 Daily dry matter intake (DMI) around the time of calving (pooled SEM = 0.85). There were no treatment effects ($P > 0.10$). Cows were either on a control treatment or supplemented with vitamin B12, folic acid, or vitamin B12 and folic acid from 3 wk before calving to 8 wk after calving. The data for wk represents the average of 7 d while the data for calving (C) was obtained from the day of calving only.

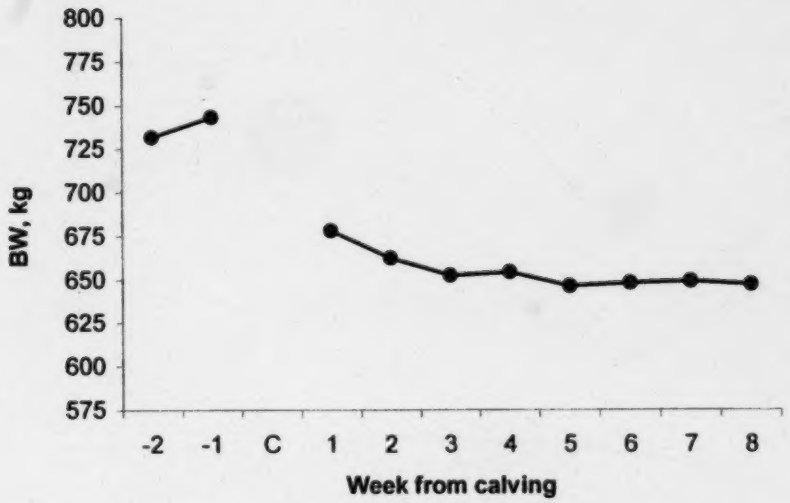

Figure 3-2 Body weight (BW) around the time of calving (pooled SEM = 10.2). There were no treatment effects ($P > 0.10$). Cows were either on a control treatment or supplemented with vitamin B12, folic acid, or vitamin B12 and folic acid from 3 wk before calving to 8 wk after calving. "C" is the day of calving.

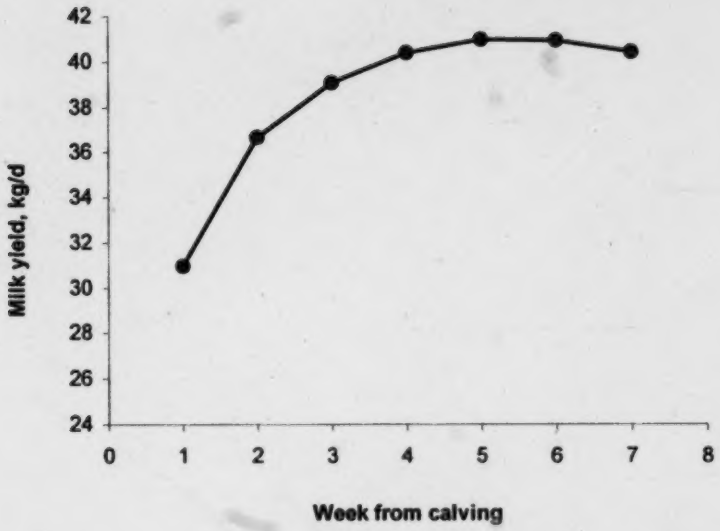

Figure 3-3 Daily milk yield during the first 7 wk of lactation (pooled SEM = 0.92). There were no treatment effects ($P > 0.10$). Cows were either on a control treatment or supplemented with vitamin B12, folic acid, or vitamin B12 and folic acid from 3 wk before calving to 8 wk after calving.

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CHAPTER 4

Evaluation of Milk Protein Synthesis as a Function of Amino Acid Supply

4.1 Introduction

With current milk pricing strategies in Canada and the U.S.A, profit margins for producers can often be improved by maximizing milk protein yield. However, increasing milk protein content by nutritional manipulation is difficult (DePeters and Cant, 1992). Increasing the supply of rumen undegradable protein (Santos et al., 1998) does not ensure improved milk protein production. Substitution of rumen degradable protein by undegradable protein can lead to decreased microbial protein synthesis and therefore no overall positive effects on the amount and profile of AA flowing to the duodenum or on milk protein output (Santos et al., 1998). Supplying AA postruminally eliminates some of these problems but the productive response to such supplementation is not always positive because intestinal and hepatic AA metabolism will have a major impact on the amount and profile of AA supplied to the mammary gland (Seal and Reynolds, 1993). Postruminal infusion of casein or AA has produced inconsistent results (Clark, 1975; Hanigan et al., 1998), although, in general, a milk protein yield increase results from postruminal AA administration. Similarly, and even to a greater extent, use of rumen-protected Met and Lys has resulted in variable milk protein yield responses (Rulquin et al., 1993; Schwab, 1996).

Among studies, the positive production responses to postruminal AA supplementation demonstrate the potential to increase milk protein yield. The challenge is to accurately predict milk protein response (Hanigan et al., 1998). The NRC (2001) predicts milk protein yield based on fixed coefficients of use of MP supply for

maintenance, gestation and milk production. The CNCPS (2000) also relates milk AA output to AA duodenal flow using fixed factors of transfer. The recovery of postruminally infused casein into milk protein averaged only 21% across 7 studies (Hanigan et al. 1998), which contrasts markedly with the 67% conversion of MP to milk protein used by NRC (2001). This strongly suggests that the efficiency of transfer of AA supply is not fixed. Indeed, it has been demonstrated that the milk transfer coefficient is not constant (Whitelaw et al., 1986). Therefore, variable efficiency should be taken into account in prediction schemes.

The current state of knowledge in the area of ruminant AA metabolism apparently does not allow for accurate prediction of milk protein yield in response to AA supply. Prediction schemes however do exist that attempt to relate AA supply to demand (O'Connor et al., 1993), and the NRC (2001) provides recommendations for lysine and methionine supply relative to total MP based on Pisulewski et al. (1996), Rulquin et al. (1993) and Schwab et al. (1992b). Rulquin et al. (2001) also proposed requirements for other essential AA based on a limited number of infusion studies.

Consequently, our first objective was to integrate data from studies in which AA were supplied postruminally and then use those data to define equations to predict milk protein yield potential and the milk protein yield response potential to AA supplementation. Our second objective was to use those same data to estimate the efficiency of AA use for lactation and requirements for EAA.

4.2 Materials and Methods

4.2.1 Source of data

Data analyzed in this study originated from 40 publications encompassing 59 trials and 213 different treatments (Appendix B). Only studies with information on feed intake and diet composition sufficient to calculate intestinal AA supply were used. To build the database, published studies involving abomasal, duodenal, or intravenous infusions of casein or free AA were selected. Trials in which rumen-protected AA were fed were excluded due to uncertainty in predicting the extent of rumen-protection of the AA.

Thirty-three of the trials involved abomasal infusions, 8 involved intravenous infusions, 17 involved duodenal infusions, and one involved intravenous and abomasal infusions. In 53 of the trials, infusions were given continuously (>20 h/d). In 4 of the trials, infusions were given over a period of less than 10 h/d, and in 2 trials, both continuous and non-continuous infusions were used. Infusions were administered on average for 11 days (s.d. = 4.8), with a range of 4 to 28 days. Two of the trials involved hyperinsulinemic-euglycemic clamps and results obtained during administration of the clamp were excluded from the dataset (4 observations; an observation refers to a treatment mean).

Of the 59 trials, Holstein (Friesian) cows were used in 52, Ayrshires were used in 5, and both Holsteins and Ayrshires were used in one. One trial did not report the breed of cow. The cows were multiparous in 58 of the 59 trials. Twenty-seven trials began in early lactation (< 84 DIM), 29 began in mid lactation (84 to 210 DIM), and one began in late lactation. Days in milk were not reported in two trials. Feed intake and production data are shown in Table 4-1. Of the 213 treatments analyzed, 57 were control treatments,

41 were casein (sodium or potassium caseinate) infusions, 9 were casein hydrolysate infusions, and 106 were free AA infusions. The content of the AA infusates ranged from 1 AA to 20 AA. (Appendix B).

4.2.2 Data analysis

All cow and diet data from the 40 publications were entered into the NRC (2001) model to obtain nutrient intakes and predictions of digestible duodenal AA flows. If individual nutrient composition was published those values were used, otherwise, default model values were used. If total diet composition was published, but not individual ingredient composition, composition of the forages was changed so that diet composition values agreed with published values. Net energy of lactation values as calculated by the NRC model were used. Model (NRC, 2001) default values for ingredient AA composition were used for all publications except one in which values were published. Intestinal digestibility of the infusates was considered to be 100% based on the NRC (2001) assumption that infused Lys and Met are 100% digestible.

Nutrient intakes, digestible duodenal AA flows, animal factors (BW, parity, DIM, etc), and experimental factors (feeding frequency, site and length of infusion, etc) were compiled into a database and tested as potential independent variables for predicting milk true protein yield (herein referred to as protein yield, PY, estimated as CP yield x 0.93 (NRC, 2001) when not published) and milk true protein yield response (herein referred to as protein response, PYR). The PYR was calculated as the difference in PY between the infusion treatments and the control treatments. The NRC (2001) model does not predict Trp or NEAA duodenal flows and so these terms were not included in any of the models tested.

Initially, PY was regressed against net energy of lactation intake (NELT = dietary supply + supply from infusion, Mcal/d), total CP supply (CPT = dietary supply + CP from infusion, g/d), total MP supply (MPT = dietary supply + MP from infusion, g/d), and MPT and NELT together. In all cases, both the linear and quadratic terms were tested. Protein yield was then regressed against the total supply (digestible duodenal flow + supplemental from the infusion, g/d) of each individual AA (expressed as AAT; for example, total supply of His is referred to as HisT) to ascertain if any one particular AA was a significant predictor of PY. Statistical analysis was performed using SAS (1999) with GLM or REG procedures.

Because no single AA was sufficient to predict PY, a model that included all AAT and NELT was then tested. The plots of PY versus the total supply of each individual AA revealed that PY increased linearly with AAT and then reached a point where increasing the supply of AAT did not result in increased PY. Identification of this breakpoint was determined with SAS (1999) using a least squares fitting method in which the intercept, slope to plateau, and plateau were estimated iteratively. The dependent variable was PY and the independent variable was individual AAT. Because the majority of the data (173 observations) lay below the breakpoint, only a linear model was tested with those data relating PY to all AAT and NELT. Laboratory was included in the model as a class variable to account for variation from factors that would be different between laboratories but not described by other factors considered in the model. Variables with the largest P-values were sequentially removed until the model contained only significant variables ($P < 0.10$). Residuals (observed - predicted) were then regressed against variables not used in the model to determine if additional terms would contribute to the

power of the model. Terms tested included ADF intake, NDF intake, non-fiber carbohydrate (NFC) intake, DIM, parity, BW, feeding frequency, and site and length of infusion. Only DIM was found to be significant and subsequently included in the model.

Similar procedures were used to develop models for the milk protein response. Our original intent was to use the data from all the publications in the hope that the statistical power would be maximized as a result of the high number of observations. However, because of the wide variation in the amount and type of infusates, it was deemed necessary to use only the studies in which casein, casein hydrolysate, or a mixture of free AA with a complete profile of EAA (with or without NEAA) was infused. The primary justification of this decision was that the infusion of only a single, or a few AA, would have the potential to skew the data and lead to erroneous conclusions. For example, if only Met was infused, and PY increased in response to the infusion, then the efficiency of other AA use would increase for a similar total input. This limited dataset contained 83 observations. Feed intake and production data are shown in Table 4-2.

Plots of PY vs. AAT generated from this limited database also revealed a breakpoint beyond which PY did not increase linearly with additional AA supply. In fact, beyond the breakpoint, PY often decreased. Figure 4-1 shows representative plots of PY vs. AA supply. Therefore, all calculations regarding PYR were performed on data in the linear portion of the plot (75 of 83 observations). Positive PYR occur beyond the breakpoint, but they are made with diminishing marginal efficiency. Because of this low efficiency, and the fact that the majority of the data lay in the linear area, the decision was made to use only the data prior to the breakpoint being reached. This decision was made

for analytical purposes only. We recognize that the relationship between PY and AAT is not truly linear followed by a plateau (rectilinear), but is in fact curvilinear.

Dietary crude protein intake (CPI), MP from dietary sources (MPI), supplemental MP from the infusions (MPS), and net energy supply arising from the diet (NELI) were each individually regressed against PYR. Individual AA were then tested to determine the degree to which any one AA was associated with PYR. For each AA, the infused amount (AAS) and the digestible duodenal flow (AAI) were regressed against the PYR. Subsequent to these models, a model containing NELI and the digestible duodenal flows of all the AA was tested. Because of the low coefficient of determination of this model, backward elimination of nonsignificant variables was not pursued.

We hypothesized that the PYR may be dependent on the pre-infusion PY, and therefore expressed PYR as a percentage change over the control value (PYRPCT). Models as described above were tested to predict PYRPCT. Additional models included 1) all AAS plus MPI, NELT and DIM; and 2) the total supply of each AA/MPT in conjunction with MPT, NELT and DIM. Because the response to supplemental AA should vary with the amount supplied in the diet, we also regressed PYRPCT against the infused amount of each AA / digestible duodenal flow of each AA ((AAS/AAI)×100). Also included in the model were NELT and MPI / MP requirement of the control animals (MPIR). The latter term was added because the response to supplemental AA is likely to vary with the cow's initial protein status (whether or not the MP requirement is already met). Removal of nonsignificant independent variables and addition of variables that weren't used in the initial model were performed as described for the PY equations.

The efficiency of CP, MP, and AA use was calculated from the same dataset used for the PYR calculations. The efficiency of use of the infused AA was calculated as the marginal change in AA output in milk protein per gram of infused AA. The efficiency of CP use was calculated as PY/CPT, and that of MP as PY/MPT. To determine the efficiency of AA use for lactation, the maintenance requirement of each AA was subtracted from the total supply to yield AA available for milk (AAFM). Efficiency was then calculated as AA yield in milk/AAFM. The maintenance requirement of each AA was calculated as the maintenance requirement for MP, as predicted by NRC (2001) multiplied by the AA composition of tissue protein as reported by O'Connor et al. (1993; Table 4-3). Because an increase in AAT without a concomitant increase in PY would result in reduced efficiency of AA use, efficiency was calculated for the data before and after the PY breakpoint.

The individual AAT that corresponded to the PY breakpoint were summed to give the total EAA needed to reach the maximal PY. Assuming that MP contains 48% EAA (NRC, 2001), the sum total of EAA was divided by 0.48 to yield MPT that resulted in maximal PY. The proportion of each EAA in MPT was then calculated, with these proportions being an indicator of what should be the relative contribution of individual AA to an "ideal" pattern of AA in MP.

4.3 Results

4.3.1 Milk protein yield

The relationship between PY and NELT was linear (Equation 1, Table 4-4), while the relationships with CPT and MPT were quadratic (Equations 2 and 3, Table 4-4). The prediction of PY was improved when MPT replaced CPT in the model but the R^2 of

< 0.7 indicates that other variables are also influencing the response. Prediction of PY was only marginally improved by including both the NELT and MPT terms (R^2 = 0.78, RMSE = 79.1).

When PY was regressed against individual AAT, the relationship was consistently better when the quadratic term was included in the model. Histidine explained the greatest amount of the variation in PY (R^2 = 0.68, RMSE = 93.6) while Val was the poorest predictor of PY (R^2 = 0.55, RMSE = 111.6). The quadratic models containing a single AAT in conjunction with NELT were an improvement (R^2 = 0.79 to 0.80, RMSE = 74.2 to 79.3) over the models containing only AAT. There was no indication that any particular AA had a unique role or was a controlling factor in determining milk protein yield.

To regress PY against the total supply of all the AA and NELT, only data below the breakpoint was used. This breakpoint was reached when the values for ArgT, HisT, IleT, LeuT, LysT, MetT, PheT, ThrT, and ValT were 110, 53, 121, 218, 165, 49, 123, 114, and 138 g/d, respectively. A linear model was generated that included the laboratory term as a class variable. This model was superior (R^2 = 0.90, RMSE = 49.0) to the models containing only one AAT and NELT. Removal of the nonsignificant terms, and addition of the DIM term resulted in an equation containing ArgT, HisT, LysT, MetT, ValT, NELT, and DIM. (Equation 4, Table 4-4). To determine if any one of the five AA had a dominant role in determining PY, PY was regressed against NELT, DIM, and each of the AA individually. In all five equations, the R^2 was greater than 0.84, indicating that PY is not dependent on only one AA but that the AA are highly interrelated.

4.3.2 Milk protein yield response

Only data below the PY breakpoint were used for the PYR calculations. The PYR averaged 77.4 g/d but varied considerably, ranging from 0 to 152 g/d. When expressed as a percentage ((treatment yield – control yield) / control yield), the mean response was 14.0%, ranging from 0% to 40%.

Correlations between PYR and MPI or CPI were very weak ($R^2 < 0.1$). Similarly, correlation between PYR and MPS was also poor ($R^2 = 0.19$). Protein yield response could not be predicted from the supply of any one AA, as expected. The relationship between PYR and individual AA, whether it was AAS or AAI, was very poor. Although the model was significant for all the AAS, the R^2 was less than 0.10 for LysS, and less than 0.32 for the remaining AA. The correlations between PYR and AAI were weaker than between PYR and AAS, with all R^2 less than 0.10. Evaluation of the protein yield response as PYRPCT improved the fit of the protein models discussed above, although the correlations with CPI, MPI, and MPS were still weak ($R^2 = 0.06$ to 0.26). Correlations between PYRPCT and individual AAS were extremely poor, with all $R^2 < 0.10$. The relationships between PYRPCT and AAI were stronger than between PYRPCT and AAS, with R^2 ranging from 0.25 to 0.34.

Because the PYRPCT is likely dependent on the AA composition of MP, a model was examined that included the ratio of each AAT in MPT in conjunction with total MPT. In this way, both the composition and the absolute amount of MP would be considered. Days in milk and NELT were also included in the model. After all nonsignificant variables were removed, Leu, Lys, Met, Thr, Val, and NELT remained in the model (Equation 5, Table 4-5). We also tested a model that included the

supplemental levels of each of the AA and MPI, NELT, and DIM. This approach was taken because of the likelihood that the response to supplementation would be dependent on the basal level of MP in the diet. Rulquin et al. (1994) noted that the response to supplemental AA varies with the CP content of the basal diet. The model had nine significant variables – Arg, His, Ile, Leu, Lys, Phe, Thr, NELT, and DIM (Equation 6, Table 4-5). The final model examined included AAS/AAI and MPIR. As previously mentioned, the MPIR term was included in the model to test whether meeting the MP requirement, as determined by NRC (2001), influences the response to supplemental AA. When the nonsignificant variables were removed, Arg, Ile, Phe, Thr, Val, MPIR, NELT and DIM remained in the model (Equation 7, Table 4-5).

4.3.3 Efficiency of amino acid use

Efficiency of use of dietary CP for the control treatments, calculated as PY/CPT, was 26.5%. When expressed on an MP basis (PY/MPT), efficiency increased to 42.6%. In nine of the 30 control treatments, the diets supplied 100% or more of MP requirements as calculated by NRC (2001). Those nine control treatments were associated with 17 infusion treatments, all of which resulted in positive PYR (mean: 67.0 g/d; range: 0.3 to 152 g/d). Marginal efficiency of the supplemental AA (marginal increase in milk AA output/AAS) was similar among the AA, ranging from 25.1% for Phe to 30.4% for Ile (Table 4-6).

The efficiency of use for lactation of either MPT or AAT considers only the MPT or AAT that is available for milk production (the requirement for maintenance has been subtracted from the total amount available). Thus, using MPT as an example, efficiency of use for lactation is calculated as PY/(MPT – MP required for maintenance). When

calculated in this manner, the efficiency of MP use for lactation was 67.1%. Because the relationship between PY and MPT or AAT was not linear, efficiencies were calculated for the data below and above the breakpoint. For MP use, efficiency was 69.3% below the breakpoint and 42.9% above the breakpoint. The efficiencies of AA use for lactation are reported in Table 4-7. Efficiencies of AA use were considerably higher below the breakpoint than above. Below the breakpoint, efficiency values ranged from 53.6% to 99.2%, while efficiencies above the breakpoint varied from 36.7 to 64.3%. Methionine was the most efficiently used AA. Efficiency values that are used in version 4 of the CNCPS (2000) prediction model are also in Table 4-7.

The AA composition of MP at the PY breakpoint is reported in Table 4-8. These values represent AA requirements as a percentage of MP. Lysine and Met, which are most commonly considered the AA co-limiting for milk protein synthesis (NRC 2001), constituted 7.26% and 2.16% of MPT at the breakpoint.

4.4 Discussion

4.4.1 Protein yield

The relationship between PY and CPT was quadratic. The NRC (2001) regressed PY against dietary CP content and DMI and found that PY increased at a decreasing rate as CP content increased. The maximum yield occurred at 22% CP. With our model (equation 2), assuming DMI of 20 kg, 23.7% CP resulted in the greatest PY. The relationship between PY and MPT was better than the relationship between PY and CPT. This is to be expected since MP is the protein actually available to the animal while CP is merely a reflection of dietary protein and not protein available at the intestinal level. Net energy of lactation intake was a better predictor of PY than was MPT. Energy intake has

a major impact on both protein and energy supply. Energy intake obviously predicts energy availability to the animal, which translates into glucose and acetate supply to the mammary gland, both of which are needed to support high levels of milk production. In addition to this, energy availability (from non-fat sources) in the rumen is a regulating factor of microbial protein yield, which usually accounts for more than 50% of the duodenal protein flux (Lynch et al., 1991; Schwab et al., 1992a). Energy content of the diet has been shown to have a greater impact on AA availability to the animal than the CP content of the diet (Reynolds et al., 1992).

The relationship between PY and individual AAT was stronger when the quadratic term was included in the model. A quadratic regression suggests that PY will increase as the intestinal AA supply increases until the point that a second AA imposes a limitation. Alternatively, it may indicate that milk protein yield has reached a plateau because of factors other than AA supply, such as the supply of other nutrients or the physiological capacity of the mammary gland.

The regression equation that explained the most variation in PY included ArgT, HisT, LysT, MetT, ValT, NELT, and DIM as significant variables. (Equation 4, Table 4-4). The positive coefficients on ArgT, HisT, LysT, and MetT indicate that as the supply of these AA increases, PY increases. Lysine and Met are often considered to be first and second limiting for milk protein synthesis (Schwab et al., 1992a; Guinard and Rulquin, 1994). The NRC (2001) suggests that the ratio of digestible Lys to Met should be 3 to 1. It is interesting to note that in the PY equation, the coefficient on Lys is approximately 1/3 the value of the coefficient on Met, so a 3 g increase in Lys supply would result in a similar PYR as a 1 g increase in Met supply, as suggested by the ratio recommended by

NRC (2001). The ratios obtained by us and by the NRC were determined from relatively independent datasets. Of the 173 treatments used in our model, only 77 treatments were used by the NRC. The NRC used an additional 30 experiments that were not included in our database. Only 31 of the 173 treatments used in our model reached the recommended Lys level, and 10 the recommended Met level. Only 6 treatments achieved both the Lys and Met required amounts. Based on these observations, a positive response to Lys and Met would be expected.

Vanhatalo et al. (1999) suggested that His was first limiting when grass silage diets were fed. In all the studies in which His was infused, there was a positive milk protein response. Although this suggests that His may be limiting, it must be remembered that in the majority of these studies, other AA were infused with His, so the increase in milk yield may actually be a response to an AA other than His. The possibility also exists that several AA are co-limiting.

In the PY equation, increases in Arg supply are associated with increases in PY. Arginine is extracted by the mammary gland in amounts two to three times greater than its output in milk (Bickerstaffe et al., 1974; Mackle et al., 2000). This suggests that its catabolism may be obligatory within the mammary gland. Arginine is a precursor for proline, whose uptake by the mammary gland is less than its output in milk (Mackle et al., 2000). The precursor role of Arg was demonstrated in the study of Bruckental et al. (1991), where mammary gland uptake of Arg was reduced by duodenal infusion of Pro, and milk protein output was increased by 16%. Arginine is also hydrolyzed to ornithine, which supplies carbon for proline synthesis and nitrogen for several NEAA (Mepham,

1982). If the metabolites synthesized from Arg have priority over its use as a constituent of milk protein, then Arg could potentially be limiting for milk protein synthesis.

The negative effect of DIM on PY can probably be attributed to the apoptosis of mammary gland epithelial cells with a concomitant reduction in protein synthetic capacity. In addition to this factor, or possibly as a result of it, is a reduction in AA uptake. Lescoat et al. (1996) stated that AA uptake by the mammary gland appears to peak at 8 weeks postpartum and decline slowly thereafter. This reduction in uptake as lactation progresses would contribute to lower milk protein yields. Istasse et al. (1986) reported that the PYR to casein was higher in early vs. late lactation, and attributed this effect to a reduction in AA uptake associated with a reduction in AA extraction rate by the mammary gland in late lactation. The reduction in AA uptake may be the result of the mammary gland's lower synthetic capacity in late lactation. The mammary gland may also have a greater response to AA supplementation in early lactation because it is likely in an AA-deficient state while in late lactation, because of its lower synthetic capacity, it is more likely to be in an AA-sufficient state. This is probably particularly true for the study of Istasse et al. (1986) because the cows were fed the same diet in the same amount at both stages of lactation. The second factor that likely contributes to the negative effect of DIM is the reduction in DMI with advancing lactation, thereby increasing the proportion of AAT devoted to maintenance.

4.4.2 Protein yield response

The mean PYRPCT from the limited dataset (described on p. 147) was 14.0%, similar to the 12% response reported by Clark (1975) for casein infusions. The response to infused casein varies with the amount and composition of the basal diet, but regardless

of diet, the response is generally positive. Clark (1975), who summarized the data from 17 studies, reported that the response to infused casein was positive in each study. Hanigan et al. (1998) reported that the mean marginal response to casein infusion was 21%, with a range of –5% to 45%.

There were only very weak correlations between PYR and MPI or CPI, indicating that the total amount of protein ingested is not the most important factor in determining a PYR. The correlation between PYR and MPS was also very weak, again demonstrating that it is not the amount of protein that regulates PYR, but more likely the AA composition of the protein in conjunction with energy availability. When PYR was regressed against the individual AAS, the correlations were very poor ($R^2 < 0.10$) for all the AA. This likely reflects the fact that the response to supplemental AA depends on the profile of AA arising from the infusate, from bacterial protein, and from rumen-undegradable dietary protein.

In the PYRPCT equations in which multiple independent variables were tested (Equations 5, 6, and 7) the prediction capability was quite high ($R^2 > 0.75$) even though only five to seven AA were included in the models. Because of this, and the fact that the AA varied among equations, the biological validity of the equations is questionable. It appears that the equations may simply be a mathematical reflection of the data, and are not representative of the metabolism of the cow. The reasons for this could be two-fold. Firstly, the number of observations is quite small; only 43 observations are used to generate the equations. Secondly, there is a substantial amount of variation in PYRPCT, ranging from 0 to 40%. The combination of these two factors would not be conducive to high correlations between PYRPCT and the independent variables. Examination of the

plots of PYRPCT and individual AA reveals the huge variation in PYRPCT for a given level of AAS or AAT.

4.4.3 Efficiency of amino acid use

The 26% efficiency factor (PY/CPT) for dietary CP is consistent with other published efficiencies (Bequette et al., 1998). Huntington (1984) reported that 30% of the nitrogen ingested was recovered in milk. In that study, 31% of the ingested nitrogen appeared in feces, and 38% appeared in urine. The large losses in the urine are attributable to the large amount of nitrogen absorbed from the gut as ammonia (52% of absorbed N), as opposed to amino nitrogen (48% of absorbed N). The ammonia is converted to urea, which is partially recycled back to the gut, but is largely excreted in the urine. The overall efficiency of MP use (for maintenance and lactation) was 43%, while the efficiency of use for lactation was 67%, coinciding with the value used by NRC (2001).

The marginal efficiency of use of the infusions (casein, casein hydrolysate, or a mixture of free AA with a complete profile of EAA) was 27%, which is similar to the 21% reported by Hanigan et al. (1998). The marginal efficiencies of use of the supplemental AA, which ranged from 25% to 30% (Table 4-6), are considerably lower than the 67% assumed by NRC (2001) for MP. The discrepancy between these two sets of values relates to the observation that the relationship between milk protein yield and MP supply is quadratic, and so, as AA supply increases, the efficiency of AA use for milk protein is reduced. This is further evidenced by the efficiency of use for lactation of the individual AA, which is not static but varies with PY and the intake of the AA (Table 4-

7). These data strongly suggest that a variable coefficient for the conversion of MP to milk protein should be used, as opposed to a fixed coefficient.

The efficiency of AA use for lactation (AA in milk/(AAT – AA for maintenance)) varies considerably among AA, ranging from a low of 0.54 for Thr to a high of 0.99 for Met. The efficiencies of the three BCAA were quite similar, ranging from 0.65 to 0.70. These values are in agreement with those used in the CNCPS model (0.62 to 0.72). The efficiency factors for His and Met, which approach 100%, are the highest of the AA, and are also in agreement with those of the CNCPS model. The uptake of Met by the mammary gland in a 1:1 ratio with its output in milk (Guinard and Rulquin, 1994 and 1995), and its minimal catabolism in the mammary gland contribute to its high efficiency rate (Mepham, 1982). The 1:1 ratio is maintained even at high levels of supplementation (32 g/d) due to decreased extraction by the mammary gland (Guinard and Rulquin, 1995). The metabolism of His, in terms of its extraction by the liver (Lapierre et al., 2000) and its uptake by the mammary gland relative to its output in milk, parallels that of Met (Guinard and Rulquin, 1995). Intuitively, one would think that AA with very high efficiencies are likely candidates to impose limitations on milk protein synthesis because the mammary gland is utilizing nearly the total amount of AA presented to it for milk protein synthesis. Indeed, both His (Vanhatalo et al., 1999) and Met (Guinard and Rulquin, 1994) have been implicated as being limiting for milk production.

The efficiency of Phe use (0.57) and Thr use (0.54) for lactation was low relative to that predicted by CNCPS (0.98). Several possible explanations exist to account for this difference. Firstly, it is possible that these AA were not limiting in the basal diets in the studies used in our database, and so additional Phe and Thr supplied by the infusions

were not incorporated into milk protein but simply catabolized. Secondly, the maintenance requirements for Phe and Thr as calculated in this study may be too low.

The efficiency of Arg use was calculated to be 0.81. This value is considerably higher than the 0.35 used by CNCPS. Considering that the mammary gland uptake to milk output ratio is in excess of 2.5:1, the validity of our efficiency factor is questionable. Several factors may contribute to our calculated efficiency being so high. The first is that it was assumed that the Arg content of muscle is representative of the Arg requirement for maintenance. This assumption may be erroneous, considering that metabolic fecal protein, which constitutes the largest part of the maintenance requirement, consists of mucous secretions, bile pigments, sloughed epithelial cells, bacterial debris, and keratinized cells (O'Connor, et al., 1993; NRC, 2001). Since the Arg content of keratin is 3.8 g/100 g of protein, only 58% of the value we used in Table 4-3 to calculate the maintenance requirement, the maintenance requirement for Arg may be lower than our prediction, and so the lactational efficiency will also be lower. The most recent release of CNCPS (version 4) uses a value of 3.3% for the Arg content of carcass tissue, which is only half the value used in the original model (O'Connor et al., 1993). If 3.3% is used for the calculation of maintenance instead of 6.6%, then the efficiency of use for lactation falls to 0.45. The second factor is that the efficiency of Arg use for maintenance, as for all the AA, was assumed to be 0.67. If the efficiency for Arg is higher, then the lactational efficiency will be lower. For example, if 0.85 is used, as in CNCPS, then lactational efficiency decreases to 0.60.

An observation of importance from Table 4-7 is that the change in AA efficiencies above and below the PY breakpoint is substantial. Current prediction schemes (NRC,

2001) use a fixed efficiency to convert MP into milk protein and this would at least partially explain why observed responses to supplemental protein are usually less than predicted (Bequette et al., 1998). At high levels of AA supplementation, efficiency is likely to be less than the 0.67 currently used for MP. This is supported by the efficiencies computed for the data above the PY breakpoint, which for all AA, were below 0.67. Both Guinard et al. (1994) and Whitelaw et al. (1986) demonstrated diminishing partial efficiencies of protein use for milk protein synthesis. In the study of Guinard et al. (1994), the efficiency of converting protein truly digested in the small intestine (PDI; after subtracting the maintenance requirement) into milk protein decreased from 0.68 to 0.48 as casein infusions increased from 0 to 762 g/d. Whitelaw et al. (1986) reported that a 200 g/d infusion of casein increased PY by 81 g/d, while a 600 g infusion only increased PY by 158 g/d. Data from these two studies, and the present study, suggest that variable, and not fixed efficiency values should be used to predict PY output relative to AA input.

Why is metabolizable protein used with diminishing efficiency for milk protein synthesis as its supply is increased? It appears to be largely due to inefficiencies within the mammary gland. Guinard et al. (1994) reported that milk output relative to mammary gland uptake of EAA decreased from 0.88 to 0.49 with increasing casein infusion. This was the result of increased extraction rates accompanied by increases in plasma AA concentrations. Diminished efficiency may also relate to metabolism in non-mammary tissues, which would reduce the AA supply to the mammary gland. With current prediction schemes (CNCPS, 2000) higher levels of digestible AA at the duodenum are required for higher milk protein synthesis because of the assumptions that AA supply to

the mammary gland would be increased, and that the efficiency of AA use for maintenance is constant. However, hepatic metabolism of AA changes in response to AA intake, and post-splanchnic supply of AA may not change in the same direction or with the same magnitude as duodenal supply. In steers given abomasal casein infusions, the increase in hepatic removal was nearly equivalent to the increase in PDV absorption so that post-splanchnic AA availability was relatively unchanged (Bruckental et al., 1997; Guerino et al., 1991). Guerino et al. (1991) suggested that the increment in extracted AA was used for hepatic protein synthesis. However, what is not known is whether the increase in protein synthesis is obligatory, or whether it occurs simply as a means to remove excess peripheral AA.

Values in Table 4-8 represent recommended AA profiles of MP for dairy cows as determined in our study. The recommendations for Lys and Met are 7.3% and 2.2% of MP, respectively. The value for Lys is similar to that recommended by NRC (2001; 7.2% of MP) and Rulquin et al. (1993; 7.3% of PDI). The value for Met is lower than the 2.4% of MP recommended by NRC and the 2.5% of PDI recommended by Rulquin et al. (1993). Perhaps the difference in values relates to the preinfusion protein status of the animals. In the study of Rulquin et al. (1993), PDI requirements were met in 88% of the trials, whereas in our database, MP requirements were only met in 30% of the trials. It is possible then that in our database Met concentrations greater than 2.2% of MP did not result in increases in PY due to limitations imposed by other AA. In the study of Rulquin et al. (1993), a higher basal level of AA would have allowed improved milk protein yields in response to Met supplementation. In terms of ration formulation, the difference

between 2.2% and 2.4% Met, as a % of MP, is substantial, and requires the use of rumen-protected Met.

The recommended allowance of 2.3% of MP for His is similar to that for Met. This value is considerably lower than that reported by Rulquin and Pisulewski (2000b), who suggested that the requirement for metabolizable His was between 3.4 and 5.6% of PDI. Considering that the hepatic uptake of His and Met are similar, that the mammary gland uptake:output ratio is similar for the two AA, and that their abundance in milk protein is similar, a value of 2.3% seems reasonable.

Milk protein yield was maximized when Leu constituted 9.6% of MP. This value is consistent with Rulquin and Pisulewski (2000a), who recommended that Leu as a percentage of PDI be between 8.87% and 11.1%. These values are both in agreement with CNCPS (2000). When that program was used to calculate the AA requirements of the average cow used in this study (567 kg, 25.6 kg milk/d), the Leu requirement was 9.4% of MPT. The allowances for the other two BCAA are also consistent with CNCPS. For the cow described above, CNCPS recommended that Ile be 5.8% of MP and Val be 6.6% of MP. These values are similar to the 5.3% and 6.1% calculated from our database. The Phe allowance of 5.4% of MP is also in agreement with Rulquin and Pisulewski (2000c), who recommended a level of Phe between 4.6% and 5.8% of PDI. Recently, Rulquin et al. (2001) have published suggested reference numbers for the EAA (Table 4-8). With the exception of His, the requirements suggested by Rulquin et al. (2001) are lower than the values obtained from our database. Whether these recommendations are significantly different will not be known until suitable feeding experiments designed to verify these values are conducted.

4.4.4 Limitations to this approach

The first objective of this study was to derive prediction equations for PY and PYR. While the regression equation for PY explained the variation in PY reasonably well, none of the PYR equations were robust enough to adequately predict the PYR. The question that needs to be addressed then is: what are the limitations to this type of approach? Why are we not able to generate equations that can predict, with a reasonable degree of certainty, the change in milk protein yield in response to different AA inputs? Decidedly, the most obvious answer in that we have an inadequate comprehension of AA metabolism in the dairy cow, particularly post-ruminally. Amino acid availability and incorporation into milk protein are dependent upon gastrointestinal metabolism, hepatic uptake, mammary gland extraction of the AA, and use of the AA for purposes other than direct incorporation into milk protein. Alterations in peripheral tissue AA availability occur due to modulation by the splanchnic tissues. These tissues dramatically change the profile of AA reaching the mammary gland. For example, the intestine uses Gln and Asp as metabolic fuels (Berthiaume et al., 2001) thereby reducing their availability to the post-splanchnic tissues. Relative to their apparent intestinal absorption, the flow of these two AA to the liver is minimal. Additionally, AA metabolism by the PDV is not a constant, but changes with diet. Seal et al. (1992) demonstrated that Gln usage by the gut was different for steers fed a forage-based diet compared to those fed a corn-based diet. As a result, animals with similar digestible duodenal AA flows may have considerably different patterns of AA entering the portal vein. Amino acids are extracted by the liver in varying proportions, thereby altering further the composition of AA reaching the peripheral tissues. For example, the liver extracts approximately 40% of the His released

by the PDV and 65% of the Phe, but virtually none of the BCAA and only small amounts of Arg (Lapierre et al., 2000).

At the present time, our understanding of the interactions between diet and splanchnic AA metabolism limits us in our ability to predict post-hepatic supply of AA, and hence, AA availability to the peripheral tissues. If this information were available, then accuracy of predictions of milk protein responses to AA supplementation would certainly be improved. Similarly, it is difficult to predict changes in mammary gland AA extraction and metabolism in response to changes in AA supply. The difficulty is further exacerbated by the differential response of the mammary gland to different AA. For example, when 762 g/d of casein was duodenally infused, mammary gland extraction of the EAA increased from 36% in the control cows to 46% in the casein-infused cows (Guinard and Rulquin, 1994). However, the response of individual AA varied greatly. The extraction of Lys and His was virtually unchanged while for Arg, Thr, Ile, Phe, and Met, extraction rates increased by 27%, 65%, 33%, 56%, and 32%, respectively. The mammary uptake:milk output ratio increased, or tended to increase, for all EAA with casein infusions.

Relative to casein infusions, the mammary gland responds quite differently when only methionine is infused (Guinard and Rulquin, 1995). As Met infusion rates increased from 0 to 32 g/d, mammary gland extraction rates decreased from 58.3% down to 10.7%, while the mammary uptake:milk output ratio remained unchanged. The uptake:output ratios of the other EAA also were unaffected by the Met infusions.

Factors that could not be evaluated in this study may influence milk protein synthesis. One of the factors that may have considerable impact on milk protein

synthesis is body protein mobilization and deposition. Additionally, genetic potential of the cow, including mammary gland size and parenchymal tissue mass, will impose upper limits to milk protein synthesis. Presumably these limits impose a nonlinear structure to the responses dependent upon how close to the limit the animal is operating. Milk protein yield is also related to endocrine status. When hyperinsulinemic-euglycemic clamps were administered in conjunction with AA infusions, protein yield was increased by 185 g/d compared to the AA infusions alone (Mackle et al., 2000). This substantial increase in PY occurred without any increase in DMI, indicating that insulin stimulated uptake of AA by the mammary gland. Environmental temperature also influences milk protein synthesis, with high temperatures reducing milk protein content (Bruhn and Franke, 1977).

A shortcoming of our approach to predict milk protein responses is the use of another prediction model (NRC, 2001) to generate duodenal AA flows. We tested the accuracy of the NRC model in predicting AA flow by using the same studies (McCarthy et al., 1989; Putnam et al., 1997; Overton et al., 1995; Klusmeyer et al., 1991a, 1991b; Schwab et al., 1992a) that Bateman et al. (2001) used in their model comparison. The mean predicted duodenal flow averaged 88% of the actual flow for all the EAA (Table 4-9) and the range in predicted vs. actual flows was considerable. Predicted flows varied from 64 to 141% of actual. This likely reduced our prediction capability because the basis of our regression equations is the duodenal AA flows.

Duodenal AA flows are in part dependent on the AA composition and ruminal degradability of the diet. In our database, default NRC (2001) values for both of these parameters were used. Considering the wide variation that exists in AA composition and

ruminal degradability within ingredients, substantial error may be associated with the AAI values.

Another potential weakness of our design is that PYR comparisons are difficult to make because of the tremendous variation betweens studies in duodenal flows of the control treatments. Rulquin et al. (1993) overcame such obstacles by creating reference production values corresponding to set levels of Lys and Met. While this approach is feasible when only two AA are being considered, it is virtually impossible when all AA are being examined. Therefore, to correct for the potential effect of variation in duodenal AA flows, AA supplementation was expressed as a percentage of the dietary duodenal flow. The approach of Rulquin et al. (1993) could not eliminate the effect of experimental factors such as stage of lactation and nutritional status, as noted by those authors. We attempted to account for these factors by two different strategies. The first approach was the inclusion of laboratory as a variable in the model. While this was significant for the PY model and improved the predictive power of the model, it was not of value in the PYRPCT models, which is expected because expressing responses relative to the control treatment encompasses the lab to lab variation. The second approach was to include the experimental factors in the models. All potential factors, such as nutrient intakes, DIM, feeding frequency, etc., were offered as independent variables in the model, but only DIM and NELT were significant.

Finally, in the studies used to predict PYR, casein or a mixture of free AA constituted the infusions. This type of infusion makes it very difficult to determine the effectiveness of a single AA in improving milk protein synthesis, and to determine the requirements for a specific AA.

4.5 Conclusion

One of our goals in assembling this database was to be able to predict the protein yield response to supplemental AA. The regression equations generated were not as robust as had been hoped, explaining less than 80% of the variation in PYR. Obviously, our understanding of AA metabolism is incomplete, particularly at the level of the mammary gland. Supplementation of AA results in changes in blood flow, mammary gland AA uptake, and partition of AA between protein synthesis and oxidation. Alterations in splanchnic metabolism also occur as a result of AA supplementation, such that duodenal supply and post-hepatic supply are not necessarily correlated. Research efforts that focus on understanding AA metabolism in these two tissues will help us predict milk production responses to varying dietary inputs.

The efficiency of metabolizable AA use for milk protein synthesis is not constant, but varies among individual AA and more importantly with PY and metabolizable AA supply. The efficiency of AA use decreases with increasing PY, such that efficiency is considerably lower after the PY breakpoint has been reached. The use of variable efficiency factors should improve our ability to predict PY in response to supplemental protein.

Amino acid allowances have been suggested, however, it must be emphasized that these allowances are based upon a limited number of observations. In spite of that, these allowances are a starting point in the determination of the AA needs of the lactating dairy cow.

Table 4-1 Dry matter intake and production of cows from all studies in the database.

	Mean	SD	Range
DMI, kg	17.7	3.3	9.1 – 27.6
DIM, d	98	53.5	7 – 220
BW, kg	567	47.3	469 – 675
Milk yield, kg/d	25.6	6.2	10.7 – 40.0
Milk protein content, %[1]	2.9	0.19	2.5 – 3.5
Milk protein yield, g/d[1]	737	166	318 – 1105
Milk protein yield response, g[2]	50.8	46.2	-66.6 – 184.1
Milk protein yield response, %[2]	8.37	8.5	-7.1 – 39.8
Milk fat content, %	3.9	0.62	2.9 – 6.0
Milk fat yield, g/d	973	182	530 – 1602

[1] True milk protein; calculated as CP × 0.93 (NRC, 2001)
[2] Difference in protein yield between treatment and control animals.

Table 4-2 Dry matter intake and production of cows from studies used in the protein yield response analyses.

	Mean	SD	Range
DMI, kg	16.2	3.6	9.1 – 27.6
DIM, d	104	50.9	7 – 220
BW, kg	552	47.3	469 – 664
Milk yield, kg/d	22.5	5.6	10.7 – 33.4
Milk protein content, %[1]	3.0	0.19	2.5 – 3.5
Milk protein yield, g/d[1]	668	164.0	318 – 1067
Milk protein yield response, g[2]	77.4	35.0	0.3 – 152.1
Milk protein yield response, %[2]	14.0	8.8	0 – 39.8
Milk fat content, %	4.1	0.66	2.9 – 5.9
Milk fat yield, g/d	895	146	530 – 1222

[1] True milk protein; calculated as CP × 0.93 (NRC, 2001)
[2] Difference in protein yield between treatment and control animals.

Table 4-3 Amino acid profile of whole empty body of cattle.

Amino acid	g/100 g of protein
Arg	6.6
His	2.5
Ile	2.8
Leu	6.7
Lys	6.4
Met	2.0
Phe	3.5
Thr	3.9
Val	4.0

from Ainslie et al., 1993.

Table 4-4 Prediction equations for milk true protein yield (PY), g/d.

Variable	n	Model[1]	R^2	RMSE	Equation
PY	213	14.37 + 25.82(NELT)	0.75	82.95	1
PY	213	-137.40 + 0.456(CPT) – 0.0000481(CPT^2)	0.50	117.7	2
PY	213	-208.53 + 0.765(MPT) – 0.000123(MPT^2)	0.69	92.86	3
PY	171	89.93 + 2.88(ArgT) + 6.93(HisT) + 0.96(LysT) + 2.73(MetT) – 3.16(ValT) + 11.99(NELT) – 0.73(DIM)	0.90	48.97	4

[1]NELT is net energy intake in Mcal/d, CPT = total dietary crude protein supply from diet and infusion in g/d, MPT is total metabolizable protein from diet and infusion in g/d, AAT = digestible duodenal AA flow + infusion in g/d, and DIM = days in milk.

Table 4-5 Prediction equations for milk true protein yield response (PYRPCT), %.

Variable	n	Model[1]	R^2	RMSE	Equation
PYRPCT	45	21.31 – 5.38(LeuT/MPT) – 13.20(LysT/MPT) + 60.04(MetT/MPT) – 30.39(ThrT/MPT) + 31.34(ValT/MPT) – 1.14(NELT)	0.76	4.77	5
PYRPCT	43	29.43 + 355.00(ArgS) – 525.62(HisS) + 209.19(IleS) – 50.30(LeuS) + 15.45(LysS) – 3.54(PheS) – 142.48(ThrS) – 1.05(NELT) – 0.05(DIM)	0.79	4.63	6
PYRPCT	43	3.81 + 2.17(ArgS/ArgI) -1.64(IleS/IleI) + 0.48(PheS/PheI) + 3.51(ThrS/ThrI) – 2.63(ValS/ValI) + 0.17(MPI/MPR) – 0.49(NELT) – 0.05(DIM)	0.79	4.56	7

[1]NELT is net energy intake in Mcal/d, MPT is total metabolizable protein supply from diet and infusion in g/d, MPS = MP supply from infusion in g/d, MPI = MP supply from diet in g/d, MPR = MP requirement in g/d, AAT = digestible duodenal AA flow + infusion in g/d, AAS = AA supplied from infusion in g/d, and DIM = days in milk.

Table 4-6 Efficiency of use of infused amino acids[1]. Efficiency values were calculated as the marginal increase in milk amino acid output per gram of infused amino acid.

Amino acid	Marginal efficiency
Arg	0.25
His	0.28
Ile	0.30
Leu	0.29
Lys	0.29
Met	0.26
Phe	0.25
Thr	0.26
Val	0.27

[1]Infusates were casein, casein hydrolysate, or a mixture of free amino acids with a complete profile of essential amino acids.

Table 4-7 Efficiency of amino acid use for lactation. Efficiency was calculated as milk amino acid output/(total amino acid supply – amino acids used for maintenance)[1].

Amino acid	Efficiency of use below PY[2] breakpoint	Efficiency of use above PY[2] breakpoint	CNCPS lactation efficiencies[3]
Arg	0.81	0.37	0.35
His	0.98	0.56	0.96
Ile	0.65	0.44	0.66
Leu	0.70	0.43	0.72
Lys	0.87	0.55	0.82
Met	0.99	0.64	1.00
Phe	0.57	0.38	0.98
Thr	0.54	0.37	0.78
Val	0.68	0.46	0.62

[1]Calculated from studies in which infusates were casein, casein hydrolysate, or a mixture of free amino acids with a complete profile of essential amino acids.
[2]PY = milk true protein yield
[3]from CNCPS (2000)

Table 4-8 Requirement for individual amino acids (% of protein) in lactating dairy cows.

Amino acid	Composition of MP[1] (%)	Composition of PDI[2] (%)
Arg	4.8	4.3
His	2.3	3.2
Ile	5.3	5.0
Leu	9.6	8.8
Lys	7.3	
Met	2.2	
Phe	5.4	5.0
Thr	5.0	4.0
Val	6.1	5.3

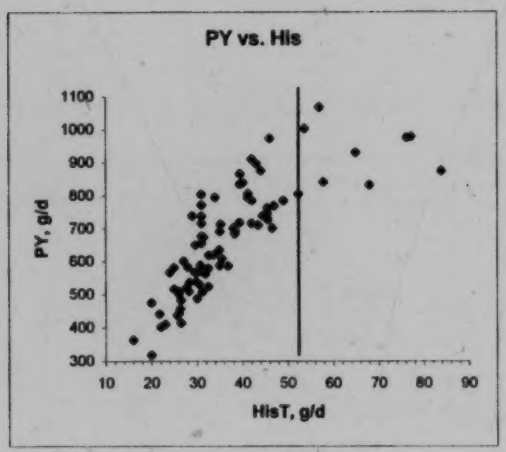
[1]MP = metabolizable protein

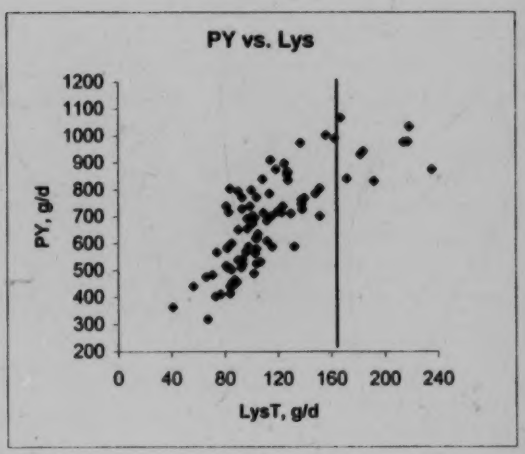
[2]from Rulquin et al., 2001; PDI = protein truly digestible in the small intestine

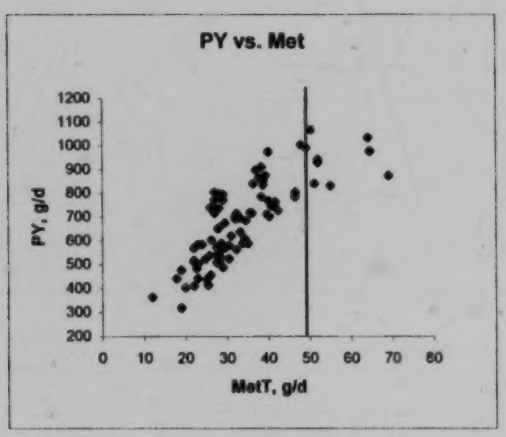
Table 4-9 NRC (2001) predicted duodenal amino acid flows vs. actual flows[1].

Amino Acid	Actual Duodenal Flow (g/d)	Predicted Duodenal Flow (g/d)	Predicted/Actual mean (%)	range (%)
Arg	145.3	125.7	87.3	65-118
His	71.0	60.3	85.2	65-116
Ile	155.1	132.4	86.2	64-111
Leu	291.1	241.7	84.7	68-116
Lys	197.9	175.2	89.9	64-126
Met	55.5	51.5	97.0	64-141
Phe	159.5	136.1	85.5	69-106
Thr	150.7	132.6	89.7	64-130
Val	182.2	149.8	83.3	66-109

[1]studies from Bateman et al., 2001







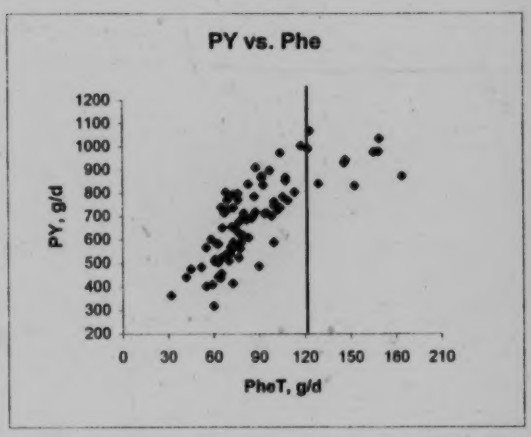


Figure 4-1 Milk true protein yield (PY) vs. amino acid supply. AAT = digestible duodenal flow + amount from infusate. The vertical line represents the breakpoint.

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CHAPTER 5

General Discussion and Conclusions

5.1 Conclusions

A number of conclusions can be drawn from the experimental work conducted in this thesis. These conclusions relate to dietary energy and protein availability to the dairy cow and the effects this has on metabolic and productive parameters, and milk protein production in response to amino acid supply. The main conclusions are:

1. Dry matter intake of multiparous cows in the immediate prepartum period is reduced by 20 to 30% relative to intake at 3 wk prepartum. The majority of this decrease occurs during the 2 to 3 d before calving. Depending on the protein and energy density of the diet, cows may be in negative protein and energy balance prior to calving. Prepartum DMI is not restricted by dietary energy density as high as 1.65 Mcal/kg.

2. Prepartum energy and protein intake higher than that recommended by NRC (1989) does not affect milk production in the subsequent lactation. Milk composition is also unaffected by prepartum energy and protein intake.

3. Physiological state (lactation vs. gestation) affects metabolites that are indicative of energy status. Plasma glucose and insulin concentrations are lower postpartum than prepartum, indicative of the high energy demands for milk synthesis. Similarly, indicators of lipid metabolism such as ACC, LPL, and FAS activity are lower postpartum than prepartum, and BHBA and NEFA concentrations are higher postpartum than prepartum. These changes indicate that a metabolic shift from lipid anabolism to catabolism occurs in early lactation in support of milk synthesis.

4. Energy density of the close-up dry diet higher than that recommended by NRC (1989) appears to reduce lipid catabolism, as evidenced by lower plasma NEFA concentrations and liver TG concentrations at calving compared to those seen in cows fed diets formulated to meet NRC specifications.

5. Body protein mobilization, as measured by plasma 3-MH concentrations, begins precalving and is more severe for cows fed low protein diets prepartum.

6. A higher protein diet in the prepartum period does not appear to be used as efficiently as a low protein diet, since PUN concentrations were higher in cows fed the high protein diets. However, this may not be a reflection of protein content as much as the AA profile of the diet. Imbalances in AA supply will result in oxidation of excess AA and urea synthesis.

7. Plasma AA concentrations during the transition period are generally not influenced by dietary energy and protein intake. The AA demand for milk protein synthesis in conjunction with the supply available from body protein mobilization likely modulates plasma AA concentrations more so than diet. In general, plasma AA concentrations decrease at calving, and recover to prepartum levels within the first few weeks of lactation. The exceptions were His, Met, and Gln, where concentrations either remained at calving levels throughout early lactation or decreased further.

8. The relationship between milk protein yield and duodenal AA supply is characterized by the presence of a breakpoint before which the relationship between protein yield and AA supply is linear and beyond which protein yield does not increase in response to additional AA supply.

9. Milk protein yield response to supplemental AA cannot be adequately predicted using the database described in chapter 4; factors other than AA supply must be influencing the response. Additionally, our prediction of duodenal AA supply may be inaccurate, which would negatively impact accuracy of predictions.

10. Efficiencies of use of EAA for milk protein synthesis differ before and after the protein yield breakpoint. Efficiencies are considerably greater before the breakpoint. Our data suggest that the use of a single efficiency value for an AA is inappropriate and may lead to underestimations of the amount of metabolizable AA required to support a given milk protein yield that is above the breakpoint. The AA commonly referred to as being the most limiting for milk protein synthesis (Lys, Met, His) are used with the highest efficiency.

11. Recommendations for Lys and Met requirements as a percentage of MP are 7.3% and 2.2%. The value for Lys parallels fairly closely the recommendation of NRC, 2001 (7.2% of MP), while that of Met is lower than the 2.4% recommended by NRC (2001). Recommendations for Leu and Phe are consistent with those of Rulquin and Pisulewski (2000a, 2000b).

5.2 General Discussion

The transition period for the dairy cow sets the stage for the upcoming lactation. If a cow suffers a metabolic disorder her milk yield for that lactation will be compromised. Therefore, it is critical that dairy producers do everything possible to ensure that cows have a smooth transition from gestation to lactation. A considerable amount of research on the transition cow has focused on prepartum energy and protein nutrition. So much emphasis has been placed on these two nutrients that the NRC (2001) now gives

recommendations for them for the close-up dry cow. Results from the research conducted for this thesis corroborate the recommendations of the NRC (2001) that 12% CP is adequate in the diet of the precalving cow, and that net energy levels in the area of 1.60 to 1.65 Mcal/kg are required (as opposed to the 1.25 Mcal/kg recommended by NRC (1989). There are several reasons for this elevated energy level.

First, DMI decreases by approximately 30% during the last week of gestation (Chapter 2, Van Saun et al., 1993; Dann et al., 1999; Greenfield et al., 2000) so increased energy density helps maintain energy intake. Maintenance of energy intake is critical to prevention of hepatic lipidosis, since cows with inadequate energy intake mobilize body fat to a greater extent thus increasing the probability of fatty liver and ketosis (Grummer, 1993). As reported in chapter 2, cows fed a high energy diet precalving had lower hepatic TG levels than cows fed a low energy diet. Cows fed the high energy diets also had higher abundance of ACC and FAS, suggesting that lipogenesis was more prevalent in these cows. The second reason for recommending energy densities in the range of 1.60 to 1.65 Mcal/kg is that fetal growth is exponential and the fetus is utilizing considerable energy during the last few weeks of gestation. Moe and Tyrrell (1972) estimated that energy requirements increase by 23% between d 250 and d 280 of gestation. Thirdly, formulation of a ration with a high energy content is generally achieved through incorporation of concentrates in the diet, which serves to adapt the rumen to the type of diet fed in early lactation. Concentrate feeding results in high propionate production in the rumen compared to forage feeding, which then stimulates papillae growth and greater volatile fatty acid absorption, thus reducing the incidence of rumen acidosis (Dirksen et al., 1985). The fourth factor which necessitates the use of

high energy diets during the close-up dry period is mammary gland growth. VandeHaar et al. (1999) suggested that the net energy required for mammary growth in late gestation is 3 Mcal/d.

In the study described in chapter 2, there were no benefits to cow productivity of feeding a diet with greater than 12% crude protein. This does not mean however that 12% is optimal since no consideration was given to AA supply. It may be possible in the future that knowledge of the cow's AA requirements would allow the use of diets with less than 12% protein. While some research efforts have focused on amino acid nutrition of the precalving cow (Bach et al., 2000; Chew et al., 1984; Wray-Cahen et al., 1997), the majority has been dedicated to the lactating cow. Reasons for this are two-fold – first, dairy producers' livelihoods are primarily dependent on the economic efficiency of milk production, so feeding management techniques that maximize milk yield while reducing feed costs, especially protein supplementation costs, will maximize profits. Secondly, agricultural sustainability is dependent on environmental sustainability, so management techniques that reduce pollutants, including nitrogen, into the environment are desirable. While a smooth transition from gestation to lactation helps the cow milk to her potential, it is proper feeding and management during lactation, especially early lactation, that ensures that her milk yield will be maximized.

Early work by Schwab et al. (1976) suggested that Lys and Met were the first limiting AA for milk production on corn-based diets. Since then, considerable research has been conducted using rumen-protected Lys and Met in an attempt to alleviate these deficiencies. Based on these research efforts, both Rulquin et al. (1993) and the NRC (2001) have published recommended levels for these two AA. The two publications are

in close agreement with one another, with the NRC recommending 7.2% and 2.4% of MP for Lys and Met, respectively, and Rulquin et al. (1993) recommending 7.3 % and 2.5% of PDI. The recommendations from chapter 4 (7.3% and 2.2% of MP for Lys and Met) are consistent with these two recommendatioins for Lys, but are lower than the recommendations for Met. Based on a review of studies in which AA had been supplied postruminally, recommendations for all EAA are provided.

The AA recommendations given by CNCPS (2000) are based on coefficients of utilization of individual AA from absorbable protein for maintenance and for milk protein synthesis. The efficiency factors for lactation for some AA differ considerably between CNCPS and those derived here. The factors used in CNCPS are based on the mammary gland uptake to milk output ratio of each AA, whereas the efficiency factors calculated here were based on the AA content in milk divided by the AA available for milk protein synthesis (the requirements for maintenance have been subtracted). The efficiency of AA use for maintenance, which was assumed to be 0.67 and equivalent to the efficiency of use of MP (NRC, 2001), may not be correct. If individual metabolizable AA are used with greater or lesser efficiency, then efficiency of use for lactation will change.

The research described herein has practical implications for dairy producers. The reported effects of prepartum dietary energy and CP levels on periparturient cow performance will aid producers in making decisions regarding formulation of prepartum diets. Additionally, the suggested recommendations for digestible AA, although based on limited data, may help producers identify AA limitations for milk protein synthesis.

5.3 Future Perspectives

The AA requirements suggested by this study and by Rulquin et al. (2001) provide a baseline from which more accurate determination of AA requirements can be made. Determination of these requirements will necessitate that each AA be tested individually. The format of this testing will likely involve supplying the AA of interest via postruminal infusion. Seventy-five percent of the animal's AA requirements, as proposed in this thesis, will be supplied through the diet. Infusions of the EAA will be given to increase the EAA supply to 110% of the proposed requirements. Nonessential AA will also be infused to ensure that a deficiency does not exist that could impose limitations on the EAA. Energy will also be supplied at 110% of requirements. The AA of interest will then be removed from the infusion in graded amounts to determine the point at which it becomes limiting. Minimal measurements that will need to be taken will include milk yield, milk protein yield, plasma AA concentration, nitrogen retention, and mammary gland AA extraction rate and uptake. While this technique is suitable within a given type of diet, the response is likely to vary between diets, even if predicted duodenal flows of AA are equivalent, because of changes in PDV and hepatic extraction of AA in response to diet. Duodenal AA flow will be a required measurement. Measurement of splanchnic AA fluxes and oxidation of a tracer AA would supply additional valuable information on AA metabolism. Other factors that need to be considered with this type of approach are the length of the infusion – will the response be the same with a 7 d infusion vs. a 28 d infusion; stage of lactation of the cows – how will the contribution of mobilized body tissue affect the response to supplemental AA; and parity of the cows – do primiparous cows have different requirements compared to multiparous cows.

Determination of AA requirements has focused on the EAA because of the assumption that NEAA are produced in adequate amounts by the cow. There has also been research that shows that the supplementation of NEAA does not improve milk or milk protein yield (Metcalf et al., 1996). However, in certain circumstances, NEAA may be limiting. For example, Gln may be limiting in early lactation, and research in this area is required. Of particular interest would be PDV and hepatic flux of Gln, other AA, and glucose. Does an increased supply of Gln have a sparing effect on glucose and other AA?

Determination of the requirements for AA for milk protein synthesis will ultimately rely on knowledge of AA splanchnic tissue and mammary gland metabolism. Acquisition of such knowledge may help us understand why an increased supply of AA to the mammary gland does not necessarily equate to an increase in milk protein synthesis. Similarly, we know that some EAA are taken up in excess by the mammary gland, but their fate is unknown. Thus, advances in the area of AA nutrition must be made to ensure the productivity and sustainability of the dairy industry. These advances will have to encompass both the productive response to AA supply as well as the biology behind the response.

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Appendix A: Analytical Procedures

A.1. Liver biopsy procedure

The biopsy tool consists of a syringe (20-50 ml), adapter, trocar, and needle.

1. Restrain the cow in a squeeze chute.
2. Clip the hair on the right side of the cow from approximately 20-50 cm from top of ribs over the last four ribs with a size 40 clipper blade.
3. Scrub surgical area on cow with Betadine surgical scrub and rinse with water. Repeat two more times.
4. Rinse surgical area with 70% ethanol.
5. Inject subcutaneously, as required, 10 ml of lidocaine (2% lidocaine hydrochloride) between the last 2nd and 3rd rib and on a line from the hip joint to the elbow.
6. Rinse with Betadine solution.
7. Make a 1.5 – 2.5 cm incision through skin over the injection area.
8. Force the biopsy tool through the peritoneal tissue toward the cow's opposite elbow to meet the diaphragm and thus the liver at a right angle.
9. Once the capsule of the liver is penetrated, remove the trocar and direct the needle in a rotary fashion into the liver. The operator should be able to feel the stroma being cut as the needle advances into the liver.
10. Place the adapter and syringe on the needle hub and apply slight suction to hold the biopsy in the needle. Several quick stabs can be made into the liver before withdrawing the needle so that it contains several cores of liver. This is necessary to ensure sufficient tissue for laboratory analysis (1 gram of tissue is required for triglyceride and glycogen analysis).
11. Withdraw the needle and flush the tissue out with a small amount of sterile saline. Rinse in sterile saline to remove excess blood and immediately place in liquid nitrogen.
12. Close the incision with two sutures (non-absorbable 3 metric, 2/0 USP surgical suture).
13. If more cows are to be biopsied, flush and rinse the needle with sterile saline and dry with sterile gauze.

Postoperative Care:
Monitor the cow every 6 hours during daylight hours for 3 days after surgery. Watch out for clostridial related problems. The routine use of antibiotics is not required. Remove sutures after approximately 10 days.

A.2. Adipose tissue biopsy

1. Restrain the cow in a squeeze chute.
2. Clip the hair on the tailhead and to one side of the tailhead (approximately 15 cm x 15 cm) with a size 40 clipper blade.
3. Scrub surgical area on cow with Betadine surgical scrub and rinse with water. Repeat two more times.
4. Rinse surgical area with 70% ethanol.
5. Apply epidural anaesthetic using 5 ml Lidocaine.
6. Rinse with Betadine solution.
7. Make a 6-8 cm incision in caudal tail fold.
8. Remove fat (approx. 15 g) with blunt/sharp dissection.
9. Close skin with #4 nylon surgical suture using simple interrupted sutures.

A.3. Nonesterified fatty acid microassay

Equipment:

micro plate reader with 550 nm filter
micro plates with lids (flat bottomed)
37°C warmroom
pipettes and tips

Reagents:

1. 50 mM phosphate buffer

3.45 g/l sodium phosphate, monobasic, dihydrate
(if you have monohydrate, use 3.05 g)
3.55 g/l sodium phosphate dibasic anhydrous

2. Wako Nefa-C Kit - # 994-75409

Prepared reagents are stable for one week when stored at 4°C.
Do not freeze or expose to direct sunlight.

a. Reagent A
Mix 10 ml of reagent A diluent with 1 vial of color reagent A. (Vials are vacuum sealed so open slowly to avoid losing powder). Gently invert the vial until contents are completely dissolved. Combine contents with 13.3 ml 50 mM phosphate buffer. Mix again and record date prepared.

b. Reagent B
Mix 20 ml of reagent B diluent with 1 vial color reagent B. Invert until contents are dissolved. Combine contents with 33.3 ml distilled water and mix again. Record date prepared.

c. Standard
Prepare serial dilutions of provided standard (1000 µmol/l) using distilled water to make the following standards: 500, 250, and 125 µM NEFA; aliquote and store at –80°C.

Sample:

serum or plasma collected on EDTA

Assay:

Run all standards and samples in triplicate. Have a standard curve, blanks (add water in place of standard) and control sample on each plate.

1. Add 10 µl sample or standard to assigned well.
2. Add 95 µl reagent A to each well.
3. Shake on the plate reader.
4. Cover and incubate in warm room for 30 minutes.
5. Add 195 µl reagent B to each well.
6. Shake on the plate reader.
7. Cover and incubate in warm room for 30 minutes.
8. Let the plate cool for a few minutes.
9. Read on plate reader at 550 nm.

If samples are hemolyzed or lipemic, sample blanks are required. Sample blanks are prepared the same way as the samples except the sample is added to the well after the first 30 minute incubation.

Calculations:

1. Perform linear regression using the standard curve (μM NEFA vs. Absorbance)
2. μM NEFA in sample is direct. If samples are above 1000 μM, dilute sample with distilled water and repeat.
3. If sample blanks are used, the absorbance of the sample blank is subtracted from the absorbance of the sample prior to the calculations being performed.

A.4. D-ß-Hydroxybutyrate assay (BHBA)

3-HB-dehydrogenase catalyzes the oxidation of BHBA to acetoacetate, with an equimolar amount of NAD reduced to NADH. The increase in absorbance at 340 nm is directly proportional to the amount of BHBA in the sample.

Equipment:

micro plate reader with 340 nm filter
micro plates with lids (flat bottomed)
37°C warmroom
pipettes and tips

Reagents:

1. 0.2 M Tris Buffer/NAD

- dissolve 4.844 g Tris (hydroxymethyl) aminomethane in ~180 ml ddH_2O, adjust to pH 9.0; make up with ddH_2O to 200 ml; store at 4°C.
- day of assay: mix 30 mg NAD (Sigma #N7004) in 15 mls buffer.

2. 3-hydroxybutyrate dehydrogenase (3-HBDH)

- Sigma #H6126
- day of assay: centrifuge 130 µl stock enzyme in microcentrifuge tube (12,000 g x 10 minutes); discard supernatant; resuspend in 1.0 ml ddH_2O.

3. BHBA standard

- Sigma #H6501
- make a 100 mg/dl stock solution: dissolve 200 mg DL- ß-hydroxybutyrate (sodium salt-racemic mixture (1/2 D, 1/2L) in 100 ml ddH_2O.
- dilute the stock to make aliquots containing 2.5, 5, 10, 25, and 50 mg/dl; store frozen.

Sample:

plasma collected on EDTA or heparin

Assay:

Run all standards and samples in triplicate. Have a standard curve, blanks (add water in place of standard) and control sample on each plate.

1. Add 150 µl Buffer/NAD to each well.
2. Add 5 µl water, standard, or sample to assigned well.
3. Shake on the plate reader.
4. Read on plate reader at 340 nm (initial reading).
5. Add 10 µl 3-HBDH to each well.
6. Shake on the plate reader.
7. Cover and incubate in warm room for one hour.
8. Read on plate reader at 340 nm (final reading).

Calculations:

Subtract initial absorbance from final absorbance. Determine values by linear regression.

A.5. Insulin assay

Reagents:

1. **Iodination: (the trace)**
   - I^{125} purchased from Amersham (IMS-130)
   - insulin purchased from Sigma #I-5500
   - dilute in 0.1 M acetic acid to obtain a concentration of 0.1 µg/µl
   - aliquot into 6 vials of 30 µl each: 3 µg/30 µl
   - final radioactivity is approx. 15,000 cpm/100 µl of BSA buffer

2. **Standards:**
   - use the same insulin as for the iodination
   - for the standard curve, make the following standards using serial dilution:
     12.5, 25, 50, 100, 200, 400, 800, and 1200 pg/100 µl
   - use BSA buffer as the diluent

3. **1st antibody:**
   - guinea pig anti-b-insulin (Linco #1010-01)
   - make up in EDTA buffer
   - dilution is 1:100 (may have to be changed depending on degree of binding)

4. **2nd antibody:**
   - goat anti-guinea pig – (Linco #2020)
   - reconstitute in 100 ml EDTA buffer:
     10 ml goat anti-guinea pig antibody
     3.34 g PEG – polyethylene glycol
     90 ml EDTA buffer
   - add the PEG drop by drop to the solution while stirring
   - make up the final volume to 100 ml

5. **NGPS – normal guinea pig serum:**
   1% - 1 ml in 100 ml EDTA buffer

6. **0.01 M PBS buffer with 1% BSA:**

NaH_2PO_4	0.01M:	1.2 g/l
NaCl	0.15M:	9.0 g/l
BSA	1% :	10.0 g/l

   - stir, adjust the pH to 7.5
   - make up the volume to 1 L
   - store at 4°C

7. **EDTA buffer**

NaH_2PO_4	0.01M:	1.2 g/l
NaCl	0.15M:	9.0 g/l
EDTA	0.05M	18.6 g./l

- heat and stir to dissolve
- adjust the pH to 7.5
- make up the final volume to 1 L.
- store at 4°C

Sample:

plasma collected on heparin

Assay:

1. put 100 µl of sample in the RIA tubes
2. add 400 µl of BSA buffer*
3. add 100 µl of the 1st antibody
4. add 100 µl of the trace
5. incubate for 24 hours at 4°C
6. add 1 ml of the 2nd antibody
7. incubate for 1 hour at 4°C
8. add 100 µl of 1% NGPS
9. incubate for 30 minutes at 4°C
10. centrifuge (20 minutes, 2800 RPM)
11. decant and count the precipitate on the gamma counter (pg/100 µl)
    Wallac 1470 Wizard Automatic Gamma Counter

* total sample and buffer = 500 µl

A.6. IGF-1 assay

Reagents:

1. **Iodination: (the trace)**
   - I^{125} purchased from Amersham (IMS-130)
   - IGF-1 purchased from Gropep – PO Box 10065 Gouger Street
     Adelaide SA 5000
     Australia
     lot EJG-CO1

   - dilute in 0.1 M acetic acid to obtain a concentration of 0.1 µg/µl
   - aliquot into 4 vials of 30 µl each: 3 µg/30 µl
   - final radioactivity is approx. 10,000 cpm/100 µl of EDTA buffer

2. **Standards:**
   - use the same IGF-1 as for the iodination
   - for the standard curve, make the following standards using serial dilution:
     6.25, 12.5, 25, 50, 150, 200, and 300 pg/100 µl
   - use EDTA buffer as the diluent

3. **1st antibody:**
   - anti-IGF-1, Somatomedin C Rabbit anti-serum – obtained from USDA (reproductive lab, Beltsville MD 20705)
   - make up in EDTA buffer
   - dilution is 1:4000 (may have to be changed depending on degree of binding)

4. **2nd antibody:**
   - goat anti-rabbit – obtained from Linco, lot RB 1008 (catalogue # 2050)
   - reconstitute in 100 ml EDTA buffer + normal rabbit serum 1:500 (0.2 ml NRS in 100 ml of buffer)

5. **7% PEG:**
   - PEG – polyethylene glycol (Sigmal # P-2139)
   - dissolve 7 g PEG in 90 ml of EDTA buffer, stir
   - make up the final volume to 100 ml
   - store at 4°C

6. **Formic Acid:**
   - make fresh daily
   - combine the following:
     5 ml formic acid
     7 ml double distilled water
     60 µl Tween-20

7. EDTA buffer: for one liter

4.14 g sodium phosphate monobasic (0.05M)
200 mg protamine SO_4
2.5 g BSA (bovine serum albumin) (0.25%)
200 mg sodium azide (0.02%)
3.72 g EDTA (0.01M)
add double distilled water, and heat and stir to dissolve
adjust the pH to 7.4 (very important) and make up the final volume to 1 L.

Sample:

plasma collected on EDTA

Assay:

Deproteinization

1. mix 100 µl plasma and 50 µl formic acid in an Eppendorf tube
2. vortex immediately
3. immediately add 350 µl acetone and vortex (this results in a 1/5 dilution)
4. centrifuge at 4°C, 6300 RPM, 15 min
5. at this point, it can be left for a few hours before doing the dilution and the curve
6. make a 1/31 dilution with the supernatant (20 µl supernatant and 600 µl EDTA buffer)
7. put immediately in the RIA tubes containing buffer

RIA procedure

1. put 200 µl EDTA buffer in the RIA tubes
2. add 100 µl of the deproteinized, diluted supernatant prepared above*
3. add 100 µl of the 1st antibody
4. add 100 µl of the trace
5. incubate for 24 hours at 4°C
6. add 100 µl of the 2nd antibody, put the PEG at room temperature
7. incubate for 1 hour at 4°C
8. add 500 µl PEG and centrifuge immediately (27 minutes, 2800 RPM)
9. decant and count the precipitate on the gamma counter (pg/100 µl)
   Wallac 1470 Wizard Automatic Gamma Counter

* total sample and buffer = 300 µl

Calculations:

- to convert pg/100 µl to ng/ml:
  pg/100 µl x 10 = pg/ml
  pg/ml / 1000 = ng/ml

  1/5 x 1/31 = 1/155
  10/1000 x 155 = 1.55
- therefore, values obtained from the gamma counter are multiplied by 1.55 to give values expressed as ng/ml

A.7. Growth hormone assay

Reagents:

1. **Iodination: (the trace)**
   - I^{125} purchased from Amersham (IMS-130)
   - GH purchased from Bioproduct (Cedar Lane) H070/H, lot 015/2
   - dilute in 500 µl 0.1 M acetic acid to obtain a concentration of 0.1 µg/µl
   - aliquot into 5 vials of 30 µl each: 3 µg/30 µl
   - final radioactivity is approx. 12,000 cpm/100 µl of BSA buffer

2. **Standards:**
   - use the same GH as for the iodination
   - for the standard curve, make the following standards using serial dilution:
     100, 200, 400, 800, 1600, 3200, and 6400 pg/100 µl
   - use BSA buffer as the diluent

3. **1st antibody:**
   - rabbit anti-b-GH – product of Agriculture and Agri-food Canada, Lennoxville
   - make up in EDTA buffer
   - dilution is 1:10,000 (may have to be changed depending on degree of binding)

4. **2nd antibody:**
   - goat anti-rabbit – obtained from Linco, lot RB 1009 (catalogue # 2050)
   - reconstitute in 100 ml EDTA buffer:
     10 ml goat anti-rabbit antibody
     3.34 g PEG – polyethylene glycol
     90 ml EDTA buffer
   - add the PEG drop by drop to the solution while stirring
   - make up the final volume to 100 ml

5. **NRS – normal rabbit serum:**
   1% - 1 ml in 100 ml EDTA buffer

6. **0.01 M PBS buffer with 1% BSA:**

NaH_2PO_4	0.01M:	1.2 g/l
NaCl	0.15M:	9.0 g/l
BSA	1% :	10.0 g/l
Thimerosal	100 mg/l:	0.1 g/l

   - stir, adjust the pH to 7.5
   - make up the volume to 1 L
   - store at 4°C

7. **EDTA buffer**

NaH_2PO_4	0.01M:	1.2 g/l
NaCl	0.15M:	9.0 g/l
EDTA	0.05M	18.6 g./l

- heat and stir to dissolve
- adjust the pH to 7.5
- make up the final volume to 1 L.
- store at 4°C

Sample:

plasma collected on heparin

Assay:

1. put 200 µl of sample in the RIA tubes
2. add 200 µl of BSA buffer*
3. add 100 µl of the 1st antibody
4. add 100 µl of the trace
5. incubate for 24 hours at room temperature
6. add 1 ml of the 2nd antibody
7. incubate for 1 hour at 4°C
8. add 100 µl of 1% NRS
9. incubate for 30 minutes at 4°C
10. centrifuge (20 minutes, 2800 RPM)
11. decant and count the precipitate on the gamma counter (pg/100 µl)
    Wallac 1470 Wizard Automatic Gamma Counter

* total sample and buffer = 400 µl

A.8. 3-Methylhistidine analysis by HPLC

Reagents:

For all solutions requiring water, use HPLC grade H_2O

1. 0.1 mM histidinol (internal standard)
- 2.1847 mg histidinol
- 100 ml ddH_2O

2. 3.0 M $HClO_4$
- 43.054 g $HClO_4$
- make up to 100 ml with H_2O

3. 1.5 M NaOH
- 6.1665 g NaOH
- make up to 100 ml with H_2O

4. 0.2 M $Na_2B_4O_7$
- 8.0416 g $Na_2B_4O_7$
- add 180 ml ddH_2O, adjust pH to 9.0
- make up to 200 ml with H_2O

5. 2.0 M HCl
- 14.59 ml concentrated HCl
- make up to 200 ml with H_2O

6. 0.1 mM 3-methylhistidine

7. 2.5 mM centyltrimethylammonium bromide

8. Solvent A
- 2.5 mM centyltrimethylammonium bromide
- 0.1 M sodium acetate buffer

pH 6.5

9. Solvent B
- 10% 2.5 mM centyltrimethylammonium bromide
- 90% acetonitrile

pH 6.5

10. Fluorescamine
- 160 mg fluorescamine
- make up to 100 ml with acetonitrile

Sample:

plasma or urine

Sample Preparation:

1. put 200 µl sample or standard in appropriately labeled tubes
2. add 100 µl 0.1 mM histidinol
3. deproteinize with 100 µl 3.0 M $HClO_4$
4. centrifuge at 1850 x G for 15 minutes

Derivatization:

1. put 200 µl supernatant (deproteinized sample) into screw cap vials
2. add 400 µl H_2O (add water to the urine samples only; not the plasma)
3. add 100 µl 1.5 M NaOH
4. add 400 µl 0.2 M $Na_2B_4O_7$
5. while vortexing, add 250 µl fluorescamine
6. allow samples to sit for 1 minute
7. add 400 µl 2.0 M HCl
8. cap the vials and vortex
9. incubate for 45 minutes at 90°C in a water bath
10. extract the samples twice with 1.5 ml of diethyl ether

HPLC:

After incubation, analyze the samples using a Varian Model 5500 Liquid Chromatograph with a Varian 2070 spectrofluorometer detector (excitation 340 nm, emission 450 nm) and a Varian 9090 autoanalyzer. Separations are completed on a 3 micron reverse phase column (Supelcosil 4.6 x 150 mm C18) using solvents A and B in a binary gradient and a flow rate of 1.0 ml/min.

A.9. Amino acid analysis by HPLC

Reagents:

For all solutions requiring water, use HPLC grade H_2O

1. **Fluoraldehyde**
   - dissolve 250 mg σ-phthaldialdehyde in 6 ml methanol
   - add 56 ml 0.04 M sodium borate buffer (pH 9.5)
   - add 250 µl 2-mercaptoethanol
   - add 2ml Brij 35

   Note: solution is stable for one week

2. **Ethanoloamine (EA) and β-amino-n-butyric acid (BABA) - internal standard**
   a. stock solution (5 µmol/ml):
      - dissolve 0.0488 g EA and 0.0515 g BABA in 100 ml H_2O

   b. working solution (25 nmol/ml):
      - 500 µl of stock solution is diluted in 100 ml H_2O

   Note: solution is stable for one week

3. **Glutamine, Asparagine, Tryptophan Standard (GAT)**
   - 0.073 g glutamine
   - 0.075 g asparagine
   - 0.102 g tryptophan
   - dissolve in 100 ml H_2O

   Note: make fresh with every run

4. **Amino Acid Stock Standard**
   - Sigma # AA-S-18

5. **Citrulline, Taurine, Ornithine Standard (CTO)**
   - stock solution is 5 µmol/ml

6. **Amino Acid Working Standard (50 nmol/ml)**
   - 500 µl amino acid stock solution
   - 250 µl GAT standard
   - 250 µl CTO standard
   - make up to 25 ml with H_2O

7. **5% w/v Trichloracetic Acid**
   - 5 g trichloroacetic acid
   - dissolve in 100 ml H_2O

8. **Solvent A: 0.1 M sodium acetate**
   - 27.2 g sodium acetate
   - add 1600 ml H_2O
   - adjust pH to 6.8
   - add 110 ml methanol
   - add 10 ml tetrahydrofuran
   - bring up the volume to 2 L with H_2O

9. Solvent B

- methanol

Sample:

plasma

Sample Preparation:

1. put 50 µl plasma or amino acid working standard in appropriately labeled tube
2. add 100 µl EA/BABA
3. vortex
4. add 200 µl trichloracetic acid
5. vortex
6. centrifuge at 2800 x G for 15 minutes
7. transfer 200 µl of the supernatant into an HPLC vial
8. add 150 µl saturated $K_2B_4O_7$ to the supernatant
9. add 350 µl H_2O

HPLC:

Place 4 or 5 standards at the start of the run, one every 10^{th} sample, and then 2 or 3 at the end of the run. Analyze the samples using a Varian Model 5500 Liquid Chromatograph with a Varian 2070 spectrofluorometer detector (excitation 340 nm, emission 450 nm) and a Varian 9090 autoanalyzer. Separations are completed on a 3 micron reverse phase column (Supelcosil 4.6 x 150 mm C18) using solvents A and B in a binary gradient and a flow rate of 1.0 ml/min.

A.10. Liver lipid extraction

Reagents:

1. **Saline (0.15M NaCl)**
   - 0.9 g NaCl
   - 100 ml dH_2O

2. **$CHCl_3$:MeOH (3:1)** – make fresh daily
   - 300 ml chloroform
   - 100 ml methanol

3. **Ethanol**

4. **0.29% NaCl**
   - 0.29 g NaCl
   - 100 ml dH_2O

Equipment:

1. 13 x 100 disposable glass tubes (3 per sample)
2. IKA Ultra-Turrax T25 homogenizer with S25N 8G dispersing tool
3. Aluminum pans for dry matter. Label and place in 60°C oven to dry before starting.
4. Acid washed 15 ml screw top glass centrifuge tubes with Teflon-lined caps (6 per sample)
5. Glass funnels, acid washed
6. Filter paper to fit funnels, Whatman #1
7. Vortex
8. Nitrogen evaporator
9. Forceps and scissors to handle tissue
10. Kimwipes

Liver preparation and homogenation:

1. Thaw liver sample (at least 0.5 g) on ice in its container.
2. Remove sample with forceps and blot off moisture on kimwipe. Put sample in a weighing pan.
3. Mince tissue with scissors to get homogenous mixture.
4. Weigh out approximately 0.1 g of liver into 13 x 100 disposable glass tubes. Do triplicates if possible.
5. Homogenize the sample in 2.0 ml saline for 20 seconds. Rinse the homogenizer dispersing tool with 2.0 ml saline and combine this to the homogenate.
6. Clean homogenizer after each sample with ethanol and then distilled water.
7. Each tube is aliquoted as follows:
   1.0 ml – DM analysis at 60°C. Save some saline to get DM on it to subtract from tissue solution
   2.0 ml – total lipid extraction

Freeze remaining sample for repeat if needed.

Sample dry matter:

1. Weigh a cooled aluminum pan and record weight.
2. Add 1 ml of homogenate (mix well before aliquoting)
3. To separate pans add 1 ml of saline used for homogenization
4. Dry at 60°C overnight.
5. Cool pan and record weight.
6. g DM/ml homogenate =
   (weight of dried sample pan – pan weight) - (dried saline pan – pan weight)

Lipid extraction: (in acid washed tubes)

1. Add 2 ml homogenate + 5 ml $CHCl_3$:MeOH to 15 ml glass tubes and cap.
2. Vortex hard for 30 seconds. Let sit at room temperature overnight.
3. Centrifuge at 20°C for 15 minutes, 1000 rpm.
4. Aspirate water layer leaving the interface and organics behind.
5. Filter entire tube through #1 paper into clean 15 ml tubes.
   Rinse sample tube with 2 ml $CHCl_3$:MeOH and decant into funnel.
   Rinse sample tube with 1 ml $CHCl_3$:MeOH and decant into funnel.
   Rinse filter paper three times with $CHCl_3$:MeOH.
6. Before filter paper dries, add 2-3 ml 0.29% NaCl using pasteur pipette to the top of the paper, forcing solvents down into the new tube.
7. Centrifuge at 20°C for 15 minutes, 1000 rpm.
8. Aspirate water layer leaving the interface and organics behind.
9. Add 0.5 ml 0.29% NaCl to each tube, vortex well and repeat steps 7-8 (centrifuge for 30 min at 1200 rpm).
10. Vortex and add methanol (2-3 ml) until solution clears
11. Dry lipid down under nitrogen, cap, and store frozen until triglyceride analysis.
12. Continue with the triglyceride assay.

Note: dried samples must never be exposed to pure air: the samples will oxidize and give false results.

A.11. Triglyceride analysis from liver lipid extract

Reagents:

1. **Chloroform:methanol 3:1**

2. **2-propanol:** optima grade (Fisher #1464-4)

3. **Activated alumina:** (Sigma # A9003) Rinse alumina with methanol in a large beaker to remove fine particles. Allow to settle and pour off methanol. Allow to dry in a hood overnight. Then spread in a thin layer in a glass petri dish. Activate by heating at 110°C overnight. Store covered at room temperature. It can be stored for a couple of weeks if kept dry. If needed, reactivate.

4. **5% KOH:** Dissolve 5 g KOH in 60 ml distilled water and add 40 ml 2-propanol. Stable at room temperature for 6 months.

5. **Sodium metaperiodate solution:** Dissolve 77 g anhydrous ammonium acetate in 700 ml distilled water. Add 60 ml glacial acetic acid and 650 mg sodium periodate. Dissolve and dilute to 1 liter with distilled water. Store dark. Stable at room temperature for 6 months.

6. **Acetyl acetone reagent:** This reagent needs to age for one day before using. Add 0.75 ml acetylacetone to 20 ml 2-propanol and mix well. Add 80 ml distilled water and mix. Store dark. Stable for one week at room temperature.

7. **Working standard:** Make a five fold dilution of Sigma glycerol standard #339-11 (250 mg/dl). Add 5 ml to a 25 ml volumetric flask and dilute to 25 ml with 2-propanol. This will yield a 0.5 mg/ml standard.

8. **Chloroform**

Equipment:

Centrifuge
2 ml and 5 ml disposable glass pipettes
15 ml glass screw top tubes with Teflon lined caps
Orbital shaker
Vortex
Nitrogen evaporator
Water bath, 60-70°C and 50°C
Spectrophotometer or plate reader for 405 nm
0.2 ml and 1 ml Pipetman with tips
repeater pipette with tips

Sample preparation:

1. Dilute dried lipid sample with 2 ml chloroform:methanol. Cap tubes tightly and vortex well. Make sure all lipid is in solution.
2. Add 8 ml chloroform and 0.4 g activated alumina to each tube. Cap tightly and shake tubes on an orbital shaker for 30 minutes at room temperature. Then centrifuge for 3 minutes at 2500 rpm.
3. Transfer 2 ml aliquot to a new 13 X 100 disposable glass tube being careful not to transfer any alumina particles.
4. Dry down with nitrogen completely.
5. Prepare standard curve. Pipette the following amounts into 13 X 100 glass disposable tubes (duplicate for each amount). Bring total volume of each tube to 2 ml with 2-propanol.

ml working standard	mg TG/tube
0	0
0.1	0.05
0.2	0.1
0.4	0.2
0.8	0.4

6. To the dry sample tubes, add 2 ml 2-propanol and vortex to dissolve.
7. Add 0.6 ml 5% KOH to all tubes and incubate at 60-70°C for 15 minutes.
8. After cooling, add 1.0 ml sodium metaperiodate and 0.5 ml acetylacetone reagent. Vortex. Incubate at 50°C for 30 minutes.
9. After cooling, read at 405 nm. Zero with blank. Can be transferred to microassay plates and read on the plate reader.

Calculations:

Perform linear regression on the standard curve. Determine mg TG in the assay tubes. If samples are off the curve, repeat using 1 ml of sample in step 3 and multiply final result by 2.

mg TG /mg liver DM =

$$\frac{(\text{mg TG})(10\text{ ml})}{(\text{ml } CHCl_3 \text{ in assay tube})(\text{ ml homogenate extracted})(\text{mg DM/ml})}$$

If everything is kept as above the equation = $\frac{(\text{mg TG})(2.5)}{\text{mg DM}}$

Multiply by 100 to express results as a percentage. Results should agree within one percentage unit, otherwise repeat.

A.12. Liver glycogen assay

Reagents:

1. **30% KOH saturated with sodium sulfate:**
   - dissolve 300 g potassium hydroxide in 1 liter distilled water
   - saturate solution by adding sodium sulfate, anhydrous, until it no longer goes into solution; some will remain on the bottom of the container.

2. **95% Ethanol**
   - mix 950 ml ethanol with 50 ml distilled water

3. **5% Phenol**
   - dissolve 50 g phenol crystals in 1 liter distilled water

4. **Sulfuric acid** – reagent grade (96-98%)

5. **Glycogen standard**
   - Make a stock solution of 5 mg/ml; dissolve 25 mg glycogen powder (Sigma G-0885) in distilled water to 5 ml

Technical Notes:
Keep liver samples on ice after removal from frozen storage. Glycogen breaks down very quickly in tissues at room temperature, therefore, extract glycogen as soon as possible after thawing. Do not use samples that have been previously thawed and refrozen.

The procedure consists of two phases - glycogen extraction followed by hydrolysis and glucose measurement.

Glycogen extraction:

1. Label and record the weight of disposable weighing pans.
2. Thaw liver tissue on ice. Remove about 1 g of tissue and blot dry with a kimwipe and place in a weighing pan. Mince the tissue with scissors.
3. Remove approximately 0.1 g of tissue and place into a glass screw-capped centrifuge tube. Record the weight of the tissue removed. Do each sample in triplicate.
4. Record the weight of tissue remaining in the pan. Dry at 60°C overnight to determine % DM.
5. Add 1.0 ml of 30% KOH solution to all tubes, making sure that the tissue is completely immersed in the solution.
6. Cap tubes and place in a boiling water bath for 30-40 minutes until a homogeneous solution is obtained.
7. Remove tubes from the water bath and place on ice until cool.
8. Add 1.2 ml 95% ethanol to all tubes to precipitate the glycogen from the alkaline digestate.
9. Keep tubes on ice for 30 minutes, then centrifuge at 840 x g for 25 minutes. Carefully aspirate the supernatant.
10. Dissolve the glycogen precipitate in 3.0 ml distilled water.

Glycogen hydrolysis and glucose measurement:

1. Prepare working standard (250 ug glycogen/ml) by making a 1/20 dilution of the stock.
2. Prepare standard curve in duplicate in plastic 17 x 100 tubes as follows

µl working standard	ml water	µg glycogen
0	1.0	0
40	0.96	10
80	0.92	20
160	0.84	40
240	0.76	60
320	0.68	80
400	0.60	100

3. Prepare sample tubes by pipetting an appropriate aliquot of the glycogen solution into new plastic 17 x 100 tubes. (The amount used will vary with the concentration of glycogen in the sample. 0.5 ml is a good starting point). Bring up to a volume of 1 ml by addition of distilled water.
4. Add 1 ml of 5% phenol reagent to all tubes.
5. Rapidly add 5 ml sulfuric acid (in 10-20 seconds) to each tube directing the stream of acid against the liquid surface rather than the side of the tube to ensure good mixing.
6. Allow tubes to sit for 10 minutes, vortex, and place in a 25-30 °C waterbath for 10-20 minutes.
7. Read absorbance at 490 nm.

Calculations:

Determine µg glycogen by comparing to a standard curve generated by linear regression. The equation used to calculate the tissue content of glycogen is:

grams of glycogen/100 g tissue =

$$\frac{A_{490}}{k} \times \frac{V}{v} \times \frac{10^{-4}}{W}$$

where A_{490} = absorbance at 490 nm
V = total volume of glycogen solution (3 ml)
v = volume of aliquot used in the color reaction
W = weight of tissue sample in grams
k = slope of standard curve

A.13. Chromium assay

Digestion solution:

dissolve 10 g of sodium molybdate dihydrate in 500 ml of a 150:150:200 mixture of distilled water, concentrated sulfuric acid, and 70% perchloric acid.
Note: do not pour water into concentrated sulfuric acid.

Procedure:

1. Weigh about 2 g of feed or fecal samples into 25 ml pyrex beakers, and ash overnight at 550°C; be sure to include a sample that does not contain chromium for use as a blank. Also weigh out and ash 0.2 g of analytical pure chromic oxide for use in the standard curve determination.

2. Allow the beakers to cool down and add 25 ml digestion solution into each beaker. For the reagent blank, put 25 ml digestion solution into a beaker. Heat the beakers on a hot plate at 300-350°C until the beaker containing the pure chromic oxide digest develops a reddish or yellowish color. Heat the beakers for a further 20 minutes.
   Note: **perform this step in the perchloric acid fumehood; wear protective eyeglasses**

3. After the samples are digested, remove the beakers from the hot plate and allow them to cool down. Transfer the sample digests into 200 ml volumetric flasks; transfer the digestion solution for the reagent blank and the pure chromic oxide digest into 100 ml volumetric flasks; make up the volume with distilled water.

4. Standard Curve Determination

   a. Take 10 ml of the 100 ml solution from step 3 and make up to 100 ml in a volumetric flask (do this for both the chromic oxide solutions and the reagent blank)
   b. Take 10 ml of the solution from (a) and make up to 100 ml in a volumetric flask.
   c. Dilute the chromic oxide solution from (b) to make the following standard solutions:

mls solution	mls water	Cr_2O_3 conc.	Cr conc.
0.5	9.5	1 ppm	0.684
1.0	9.0	2 ppm	1.368
1.5	8.5	3 ppm	2.052
2.0	8.0	4 ppm	2.736
3.0	7.0	6 ppm	4.104
3.5	6.5	7 ppm	4.788
4.0	6.0	8 ppm	5.472

   d. Zero the spectrophotometer with the reagent blank solution from (b)
   e. Determine the atomic spectrophotometric absorbance of the standard solutions above with Atomic Absorption Spectrophotometer Perkin Elmer 4000, which uses an air-acetylene flame to determine chromium.
   f. Establish a regression equation to calculate the chromic oxide concentration in the feed or fecal samples.

5. Sample Chromium Determination

a. Take a 10 ml subsample from each flask in step 3 and centrifuge at 3000 rpm for 10 minutes at 20°C.

b. Take 1 ml from (5a) and make up to 5-8 ml with water. For fecal samples, use a 5x dilution; for feed samples, use an 8x dilution.

c. Determine the absorbance as in (4e).

d. Determine the chromium concentration using linear regression.

A.14. Cytosolic protein isolation

Stock Solutions:

1. **100 mM DTT (dithiothrietal, Sigma D-5545)**
   0.1542 g in 10 ml sterile ddH_2O

2. **500 mM PMSF (phenyl methyl sulfonyl fluoride, Sigma P-7626)**
   0.4355 g in 5 ml DMSO (dimethyl sulfoxide)
   - make fresh as required

3. **Aprotinin (Sigma A-1153)**
   0.005 g in 5 ml DMSO
   - aliquot and store at –20°C

4. **Leupeptin (Sigma L-2884)**
   0.0053 g in 5 ml ddH_2O
   - aliquot and store at –20°C

5. **Pepstatin A (Sigma P-4265)**
   0.005 g in 5 ml ddH_2O
   - aliquot and store at –20°C

6. **Stock Homogenization Buffer: (concentrations shown are the final concentration in the buffer)**

0.05M Tris HCl	3.94 g
0.25 M Mannitol	22.78 g
1 mM EGTA	0.1902 g
1mM EDTA	0.1463 g
5 mM Na pyrophosphate	1.1153 g
50 mM NaF	1.0498 g

- make up to 450 ml with sterile ddH_2O
- cool in frig overnight
- adjust pH to 7.5 and make up to 500 ml

Working Homogenization Buffer:
45 ml pH-adjusted stock homogenization buffer
500 µl DTT stock
100 µl PMSF stock
200 µl Aprotinin stock
200 µl Leupeptin stock
200 µl Pepstatin stock
- make up to 50 ml with stock homogenization buffer
- make on the day the buffer will be used

7. Stock Resuspension Buffer:

0.1 M Tris HCl	0.394 g
1 mM EDTA	0.0073 g
1 mM EGTA	0.0095 g
50 mM NaF	0.0525 g
5 mM Na pyrophosphate	0.0558 g

- make up to 20 ml with sterile ddH_2O
- cool in frig overnight
- adjust pH to 7.5 and make up to 25 ml

Working Resuspension Buffer:

9 ml stock resuspension buffer
40 µl Aprotinin stock
40 µl Leupeptin stock
40 µl Pepstatin stock
- make up to 10 ml with resuspension buffer

Procedure:

1. Weigh 1.0 – 1.2 g of ground adipose tissue (use dry ice to keep samples frozen all the time)
2. Add 3 ml homogenization buffer and homogenize tissue for 30 sec. using Polytron or Tekmer homogenizer
3. Centrifuge at 14,000 g (11,000 rpm; Beckman J2-21, rotor JA020.1) for 15 min.
4. Transfer the supernatant to ultracentrifuge tube carefully (avoid taking fat into the tube).
5. Centrifuge at 105,000 g (40,000 rpm; Beckman L8-70M Ultracentrifuge, rotor 50.1) for 75 min.
6. Transfer 200 µl of the supernatant into microfuge tubes for Western blot and protein content analysis and freeze.
7. Transfer the rest of the supernatant to 10 ml Falcon tube; discard the pellet.
8. Add 0.6 ml 50% PEG to the supernatant in the Falcon tube (final concentration of PEG is 10%) and vortex for 2 min.
9. Centrifuge at 11,000 g for 10 min.
10. Aspirate the supernatant and add 200 µl resuspension buffer to the pellet; resuspend the pellets gently with a glass rod.
11. Divide the solution equally into 2 microfuge tubes for ACC activity and FAS activity.

A.15. Quantitation of protein

Cytosolic protein is quantitated using BCA Protein Assay Reagent kit (Pierce, Rockford, IL, USA)

Equipment:

micro plate reader with 540 nm filter
micro plates with lids (flat bottomed)
37°C warmroom
pipettes and tips

Procedure:

1. Prepare BSA standards: 2.0, 1.5, 1.0, 0.75, 0.5, and 0.25 mg/ml.
2. Mix Reagent A with Reagent B in a ratio of 50:1.
3. Dilute sample protein 1:20 with homogenization buffer.
4. Add 10 µl of standard or sample to assigned well.
5. Add 200 µl of the reagent mixture to each well.
6. Cover and incubate at 37°C for 30 min.
7. Read on plate reader at 540 nm.

Calculations:

1. Perform linear regression using the standard curve (µM protein vs. absorbance)
2. If sample blanks are used, the absorbance of the sample blank is subtracted from the absorbance of the sample prior to the calculations being performed.

A.16. Western blotting

Reagents:

1. 1.5 M Tris-HCl, pH 8.8
18.2 g Tris base in 90 ml ddH_2O
- adjust pH to 8.8 with 10 N HCl
- make up to 100 ml with ddH_2O

2. 0.5 M Tris-HCl, pH 6.8
6.05 g Tris base in 60 ml sterile ddH_2O
- adjust pH to 6.8 with 10 N HCl
- make up to 100 ml with sterile ddH_2O

3. 30% Acrylamide
8.76 g acrylamide
0.24 g bisacrylamide
- make up to 30 ml with water
- store in cool, dark place for up to 1 week

4. 5 x Loading Dye
4 ml glycerol
1.7 ml 1.5 M Tris-HCl, pH 8.8
0.8 g SDS
2 ml ß-mercaptoethanol
0.2 g bromophenol blue
- store at -20 °C

5. 5 x Electrode Running Buffer (Stock Solution)
15.1 g Tris base
72.0 g glycine
5.0 g SDS
- make up to 1 L with ddH_2O
Note: 1 x is the working solution

6. 10% Ammonium Persulfate
50 mg ammonium persulfate
- make up to 500 ml with ddH_2O
Note: make fresh as required

7. Towbin's Transfer Buffer, pH 8.3
12.1 g Tris base
57.6 g glycine
800 ml methanol
1 ml 20% SDS
- make up to 4 L with ddH_2O and store at 4°C

8. PBS, pH 7.4

32 g NaCl
0.8 g KCl
5.76 g Na_2HPO_4
0.88g KH_2PO_4
- make up to 4 L with ddH_2O and adjust pH to 7.4 with concentrated HCl

9. TBS, pH 7.4

32 g NaCl
0.8 g KCl
12 g Tris base
- make up to 4 L with ddH_2O and adjust pH to 7.4 with concentrated HCl

10. TBST

0.01% Tween 20 in TBS

Procedure:

A. Casting the Separating and Stacking Gel

1. Prepare gel casting apparatus for Mini-PROTEAN II gel unit (BioRad Laboratories, Mississauga, ON). Prepare glass plates by washing them with absolute ethanol. Place two 1.5 mm spacers between the long and short glass plates and clamp into the clamp unit. The glass plates and spacers must be flush at the bottom in order to ensure a tight seal in the casting apparatus. Place the clamp unit in the casting apparatus in preparation for pouring the gel.

2. Before pouring the gel, ensure that there is a tight seal between the glass plates and the rubber gasket on the casting apparatus by flushing the glass plates with water and determining if there is any leakage.

3. Prepare the 5% separating gel:
   11.2 ml ddH_2O
   5.0 ml 1.5 M Tris-HCl, pH 8.8
   3.3 ml 30% acrylamide
   100 µl 20% SDS
   - degas on vacuum on low speed of stirrer for 10 min.
   - transfer to beaker and add 200 µl 10% ammonium persulfate and 16 µl TEMED - swirl

4. Pour the separating gel between the glass plates using a pasteur pipette to 1 cm from the top of the smaller plate.

5. Overlay the gel with isopropanol.

6. Let the gel polymerize for 30 min, and then rinse the isopropanol off the top of the gel with water.

7. Prepare the 4% stacking gel:
   9.2 ml ddH_2O
   3.75 ml 0.5 M Tris-HCl, pH 6.8
   2.0 ml 30% acrylamide
   75 µl 20% SDS

- degas for 10 min.
- transfer to beaker and add 75 µl 10% ammonium persulfate and 10 µl TEMED - swirl

8. Remove all of the water from the top of the separating gel.

9. Pour the stacking gel between the glass plates using a pasteur pipette.

10. Place a 15 well 1.5 cm comb into the stacking gel.

11. Add more stacking gel as the gel shrinks around the combs to ensure that the volume of the combs is maintained.

11. Let the gel polymerize for 30 min, and then remove the combs.

12. Overlay with 1 x running buffer in order to prevent oxidation of the acrylamide.

B. Sample Preparation

1. Dilute samples with homogenization buffer and 5 x loading dye so that the final concentration of protein is 1 mg/ml and the final dye concentration is 2.5 x.

2. Heat the samples in a boiling water bath for 5 min. to denature the protein.

3. Quench the samples on ice to cool them quickly.

4. Centrifuge the samples momentarily to collect the solution at the bottom of the tube.

5. Prepare the rainbow high molecular weight marker (Amersham International plc, Buckinghamshire, England) by combining 10 µl of the rainbow marker with 2.5 µl of 5 x loading dye and heating the marker in a boiling water bath for 10 min. Cool the marker on ice and centrifuge momentarily.

C. Electrophoresis

1. Place the gels into the holder for the gel unit.

2. Load the volume of sample appropriate for the amount of protein that is required for detection (25 µg of protein).

3. Place the holder into the gel unit and fill the unit with 1 x electrode running buffer sufficient to cover the electrodes and the bottom of the glass plates.

4. Using a bent pasteur pipette, remove the bubbles from the bottom of the glass plates to ensure even voltage across the gel.

5. Fill the reservoir between the 2 gels with 1 x electrode running buffer.

6. Run the gel for 1.5 h at 100 V.

D. Transfer of the Protein to the Membrane

1. Prepare the NitroPure (Micron Separations, Inc., Westborough, MA, USA) membrane, fibre pads, and the 3 mm Whatman chromatography paper by soaking them in 4°C Towbin's buffer.

2. Cut the bottom right corner of the gel before removing it from the glass plates in order to determine the orientation of the gel. Gently remove the gel from the glass plates by slowly moving the glass plate in the Towbin's buffer until the gel slides off the plate.

3. Equilibrate the gels for transfer by soaking them in 4°C Towbin's buffer for 30 min. on a shaker.

4. Place a fibre pad on the black surface of the gel holder. Cover with Towbin's buffer and then roll the pad with a small glass tube to remove air bubbles.

5. Place a piece of 3 mm Whatman chromatography paper cut to the same size of the gel on the surface of the fibre pad and roll out the air bubbles.

6. Float the gel onto the membrane in the Towbin's buffer. Place the gel face down on the filter paper. Remove the membrane, overlay the gel with Towbin's buffer, and roll out the air bubbles very gently.

7. Place the membrane face down onto the gel. Roll out the air bubbles.

8. Cover the membrane with another piece of filter paper. Roll out the air bubbles.

9. Place the second fibre pad over the filter paper and roll out the air bubbles.

10. Close the gel holder so that the grey side of the gel holder is on the top.

11. Place the gel holder in the Mini Trans-Blot (BioRad Laboratories) so that the black side of the gel holder is in contact with the anode and the grey side is in contact with the cathode. The transfer of the proteins from the gel to the membrane is from the negative to the positive electrode.

12. Perform the transfer at 300 mA for 2 h or 40 mA overnight.

13. Following the transfer, confirm the transfer by incubating the membrane in Ponceau S stain for 5 min on a shaker. Destain the membrane with distilled water. Stain the gel with Coomassie blue for 5 min on a shaker to determine whether there is any protein left on the gel. The gel is then destained with distilled water until the majority of the stain has been removed. If the transfer is poor or incomplete, the membrane will be discarded and the procedure repeated.

14. Store the membranes at 4°C until they are incubated with the antibody.

E. Antibody Incubation

E.1. ACC

1. Wash the membranes in 2 changes of PBS for 5 min each.

2. Incubate the membranes in 100 ml 10% Blotto (skim milk in TBST) for 1 h 15 min to block non-specific binding

3. Rinse the membrane twice with TBST.

4. Wash once in TBST for 5 min.

5. Incubate the membrane with the primary antibody in the appropriate concentration for 1 h (1:500 Steptavidin (20 µl Streptavidin in 10 ml TBST))

6. Rinse the membrane twice with TBST.

7. Wash in 3 changes of TBST for 20 min each.

8. Incubate the membranes for 1 min. in ECL detection reagent (Amersham International plc) prepared by combining 10 ml of Reagent 1 with 10 ml of Reagent 2.

9. Place the membranes in a heavy plastic bag and remove the excess reagent.

10. Quickly place the membranes in an x-ray cassette with Biomax S film (Kodak) and expose the film to the membranes for a length of time appropriate to generating a signal for analysis (15 s to 1 min).

11. Develop the film and then analyze the results using an imaging densitometer (BioRad Laboratories).

E.2. FAS

1. Wash the membranes in 2 changes of PBS for 5 min each.

2. Incubate the membranes in 100 ml 10% Blotto (skim milk in TBST) for 1 h to block non-specific binding

3. Rinse the membrane 3 times with TBST.

4. Wash once in TBST for 10 min.

5. Incubate the membrane with the primary antibody in the appropriate concentration for 1 h 30 min (1:250 anti-FAS (20 µl anti-FAS in 5 ml TBST)

6. Rinse the membrane three times with TBST.

7. Wash in 3 changes of TBST: 1 x 20 min, 1 x 10 min, 1 x 5 min.

8. Incubate the membrane with the secondary antibody in the appropriate concentration for 1 h 30 min (1:500 anti-mouse IgG (10 µl anti-mouse IgG in 5 ml TBST)

9. Rinse the membrane three times with TBST.

10. Wash in 3 changes of TBST: 1 x 20 min, 1 x 10 min, 1 x 5 min.

11. Incubate the membranes for 1 min. in ECL detection reagent (Amersham International plc) prepared by combining 10 ml of Reagent 1 with 10 ml of Reagent 2.

12. Place the membranes in a heavy plastic bag and remove the excess reagent.

13. Quickly place the membranes in an x-ray cassette with Biomax S film (Kodak) and expose the film to the membranes for a length of time appropriate to generating a signal for analysis (15 s to 1 min).

14. Develop the film and then analyze the results using an imaging densitometer (BioRad Laboratories).

A.17. Acetyl-CoA carboxylase activity assay

Reagents:

1. Tris Acetate (1 M)
2.718 g in 15 ml ddH_2O

2. BSA – fatty acid free
0.495 g in 15 ml ddH_2O

3. Mg Acetate (132 mM)
0.4285 g in 15 ml ddH_2O

4. K-citrate (264 mM)
1.212 g in 15 ml ddH_2O

5. $NaHCO_3$ (300 mM)
2.52 g in 15 ml ddH_2O

6. β-mercaptoethanol (20 mM)
21.48 µl in 15 ml ddH_2O
Note: prepare in fume hood

7. ATP (70 mM)
0.1929 g in 5 ml ddH_2O

8. Acetyl-CoA (5 mM)
25 mg in 6.18 ml ddH_2O

9. Preincubation Buffer
2 ml Tris acetate
1 ml BSA
1.25 ml Mg acetate
1.25 ml K citrate
500 µl β-mercaptoethanol
4 ml ddH_2O

10.. Incubation Buffer
1 ml Tris acetate
500 µl BSA
500 µl ATP
250 µl β-mercaptoethanol
625 µl Mg acetate
625 µl K-citrate
3.5 ml Acetyl-CoA
5.0 ml ddH_2O

11. Labelled $NaHCO_3$ solution
100 µl cold $NaHCO_3$ (300 mM stock)
5 µl hot $NaHCO_3$ (= 5 µCi)
45 µl ddH_2O

12. 10% Perchloric Acid

Procedure:

1. Preincubate 40 µl of sample and 40 µl of preincubation buffer for 30 min. in 37°C waterbath. Prepare blank tubes using water and buffer.
   Note: the 40 µl of sample is a combination of water and sample to supply 140 µg of protein (determined previously using BCA kit).
2. Prepare incubation tubes: put 120 µl of incubation buffer and 15 µl of $NaHCO_3$ in glass tubes, and incubate for 5 min. in 37°C waterbath.
3. Add 30 µl preincubated sample (do duplicates of each tube) into incubated buffer and let it react exactly 5 min. in 37°C waterbath.
4. Stop the reaction by adding 25 µl 10% perchloric acid.
5. Vacuum the reacted samples in a dessicator with connected water flow for 30 min. to release $^{14}CO_2$.
6. Transfer into 7 ml scintillation vials and dry under nitrogen on 60°C hot plate.
7. Add 100 µl ddH_2O and vortex well.
8. Add 4 ml scintillation fluor, and count the samples for 10 min. Also count 15 µl of labelled bicarbonate solution.

Calculations:

Molar concentration of bicarbonate in assay mixture:
(10 µl x 300 mM)/165 µl = 18.18 mM (18.18 µmol/ml)

$NaHCO_3$ amount in final incubation mixture is:
18.18 µmol/ml x 0.165 ml = 3 µmol

0.5 µCi of $NaHCO_3$ is added giving rise to 0.165 µCi/µmol.

Specific activity (SA) = 0.165 µCi/µmol x dpm of bicarbonate solution = dpm/ µmol
Divided by 1000 = dpm/nmol

ACC assay reaction product counted in scintillation counter:
= Y dpm per 5 min. reaction time
corrected for blank = Yb dpm per 5 min. reaction time

nmol $Na^{14}HCO_3$ incorporated into malonyl-CoA per minute per mg protein is calculated as:

(Yb dpm / (5 x SA) / µg protein) x 1000

A.18. Fatty acid synthase activity assay

Reagents:

1. **KPB Buffer**
   - 27.218 g KH_2PO_4
   - 0.116 g EDTA
   - make up to 100 ml with ddH_2O
   - heat to dissolve

2. **Acetyl-CoA (Sigma A2181; 0.35 mM)**
   - 0.003 g in 10.5 ml ddH_2O
   - keep frozen

3. **Malonyl-CoA (Sigma M4263; 2 mM)**
   - 0.009 g in 5.25 ml ddH_2O
   - keep frozen

4. **NADPH (Sigma N7505; 4 mM)**
   - 0.01 g in 3.0 ml ddH_2O
   - keep frozen
   - Note: photosensitive and temperature sensitive; aliquot and wrap in foil immediately

5. **β-mercaptoethanol (20 mM)**
   - 21.48 µl in 15 ml ddH_2O
   - Note: prepare in fume hood

6. **Preincubation Buffer**
   - 2.7218 g KH_2PO_4
   - 0.0308 g DTT
   - make up to 10 ml with ddH_2O

7. **Buffer Mixture for Analysis (100 samples)**
   - 25 ml KPB buffer
   - 10 ml Acetyl-CoA
   - 5 ml Malonyl-CoA
   - 5 ml β-mercaptoethanol
   - 45 ml ddH_2O
   - Note: keep solution on ice

Procedure:

1. Incubate 40 µl of sample and 120 µl of preincubation buffer for 30 min. in 37°C waterbath. Stagger the samples by 10 min intervals because 10 min. are required to read the 2 replicates on the spectrophotometer.
   Note: the 40 µl of sample is a combination of water and sample to supply 105 µg of protein (determined previously using BCA kit).
2. Combine 50 µl of the above enzyme sample with 900 µl of incubation buffer in each of 3 eppendorf tubes (2 sample tubes and 1 blank tube)
3. Transfer to cuvettes; in the blank (reference) cuvette, add 50 µl ddH_2O, mix well, and put in the reference position in a spectrophotometer with a 37°C heater block (Varian Cary UV-visible automated spectrophotometer); put one of the sample cuvettes in the measuring position.
4. Zero the spectrophotometer.
5. Add 50 µl 4 mM NADPH into the sample cuvette and mix well.
6. Immediately read the absorbance at 340 nm for a period of 3 min.
7. Record the slope of the absorbance curve.

Calculations:

$$\Delta C = \Delta A / E$$

Where ΔC = change in concentration of NADPH, ΔA = change in absorbance and E = extinction coefficient of NADPH ($E_{340nm} = 6.22\ mM^{-1}\ cm^{-1}$).

Ex: In 1 ml assay volume, if ΔA of NADPH is 0.0139/(min.cm), then,

$$\Delta C = 0.0139/6.22\ mmol/L\ min^{-1} \times 1000\ ml/L$$

$$= 0.002235\ mmol/ml\ min^{-1}$$

FAS activity = 2.234 nmol/min

FAS activity per mg protein in assay:

$$= (2.234\ nmol\ min^{-1} / \mu g\ protein\ in\ assay) \times 1000$$

A.19. Lipoprotein lipase activity assay

Reagents:

1. **3:2 Hexane:Isopropanol (HI)**

2. **7:2 Hexane:Isopropanol (HI)**

3. **Triolein**
   1 g Triolein (Sigma T-7140 or T-7502)
   - make up to 9 ml with toluene
   - store at 4°C

4. **Lecithin**
   0.2 g lecithin (Sigma P9671)
   - make up to 9 ml with chloroform
   - store at 4°C

5. **Tris Buffer**
   11.63 g Tris
   3.5 g NaCl
   - add 380 ml ddH_2O and adjust pH to 8.6
   - make up to 400 ml with ddH_2O

6. **10% BSA (Sigma A3803)**

7. **20% BSA-Tris buffer**

8. **10% BSA-Tris buffer**

9. **^{3}H Triolein (0.5 mCi/ml)**
   1mCi [9,10-3H(N)] triolein
   - make up to 2 ml with toluene
   - store at 4°C

10. **^{14}C Palmitate (0.1 mCi/ml)**
    1mCi [1-14C] palmitic acid
    - make up to 10 ml with toluene
    - store at 4°C

11. **KCl**
    5.59 g KCl
    - make up to 500 ml with ddH_2O
    - store at 4°C

12. **Heptane:Methanol:Chloroform (HMC)**
    273 ml heptane
    385 ml methanol
    342 ml chloroform

13. Alkaline Buffer

12.66 g $K_2B_4O_7.4H_2O$
27.56 g K_2CO_3
- make up to 1 L with ddH_2O; adjust pH to 10.2

14. HD Serum

- collect serum from lactating cows
- denature serum LPL by incubating for 30 min in a 60°C water bath
- centrifuge at 40,000 x g for 20 min
- store supernatant at 4°C

15. Scintillation fluid

Procedure:

A. Tritiated Triglyceride Purification

1. In a 20 ml screw capped test tube, add 0.08 ml tritiated triolein, 10 ml 3:2 hexane:isopropanol, and 2 ml alkaline buffer.

2. Vortex and allow 2 phases to separate.

3. Transfer the supernatant into another 20 ml screw capped test tube washing the meniscus with 5 ml 7:2 HI.

4. Add 5 ml Tris buffer to supernatant and vortex.

5. Allow phases to separate and transfer supernatant containing the purified triglyceride to a scintillation vial.

B. Preparation of Premixes

1. Pipette 2 ml triolein and 1 ml lecithin into the scintillation vial containing the purified tritiated TG (TG premix).

2. Pipette 2 ml triolein and 1 ml lecithin into another scintillation vial containing 0.05 ml ^{14}C Palmitate (FA premix).

3. Evaporate solvents from both vials under nitrogen.

4. Add 12.5 ml Tris buffer and sonicate on ice with a microtip at 75 watts for 3 x 1 min with 1 min pauses.

5. Add 12.5 ml of 20% BSA-Tris solution to the sonicated solution and mix.

6. Store the TG premix and FA premix at 4°C for up to 2 weeks, mixing each time before using.

C. Assay Setup

1. Setup 10 x 75 mm assay tubes on ice and add TG premix and HD serum for the sample tubes, and premix and HD serum + NaCl or 10% BSA for the blanks. Preincubate tubes at 37°C for 20 min to allow apoCII from the serum to associate with the triolein substrate.
   Note: each concentration of TG and sample needs to have blanks – either BSA, HD serum + NaCl or both – run with the samples (see table).

2. Put tubes on ice until addition of tissue homogenates.

3. To determine FA recovery factor (RF), set up in triplicate assay tubes containing 100 µl FA premix and 10% BSA or HD serum or HD serum + NaCl (depending on which blanks are run with the samples).

4. For converting counts to moles FA, pipet in 20 ml glass scintillation vials 10 µl of FA premix in triplicate and TG premix in triplicate. Add 10 ml scintillation fluid to vials and count with assay.

D. Tissue Homogenate Preparation

1. Weigh 0.5 g tissue into 15 ml Corex tubes.

2. Add 2 ml cold 0.15 M KCl and keep on ice.

3. Homogenize tissue for 10 sec with a Brinkmann Polytron homogenizer at 75% full power. Wash polytron between homogenizations in 3 L hot distilled water and then 3 L cold distilled water.

4. Centrifuge for 10 min at 3000 x g at 4°C.

5. Pour supernatant through glass wool and store on ice for no more than 2 h.

E. Incubation and FA extraction

1. To assay tubes on ice add tissue homogenate and 0.15 M KCl for a combined added volume of 40 µl.

2. After vortexing, incubate assay tubes at 37°C in shaking water bath for 1 h.

3. Terminate assay by placing tubes on ice and adding 1.65 ml HMC solution and 0.45 ml alkaline buffer. Vortex each tube for 10 sec.

4. Centrifuge tubes at 1750 x g for 15 min at 20°C.

5. Transfer 0.5 ml supernatant into 20 ml glass scintillation vials with 10 ml scintillation fluid and use appropriate counter settings for counting tritium and ^{14}C samples. Along with assay vials, count blank vials containing only 10 ml scintillation fluid along with ^{14}C and tritium samples.

F. Calculations

Calculate DPM for tritium and ^{14}C samples after subtracting DPM for appropriate blank scintillation vial.

LPL fatty acid recovery factor = DPM per tube / (DPM per 10 ul FA premix x 10)

DPM/nmol FA = DPM premix/300

nmol/g tissue = ((DPM sample tube/DPM RF) - (DPM sample blank/DPM RF blank) / DPM/mnol FA

Description	Premix	10% BSA-Tris	HD + NaCl	BSA	HD serum	KCl	Homog
Salt blank, 5 mM TG	100	0	60	0	0	20	20
BSA blank, 5 mM TG	100	0	0	60	0	20	20
LPL act., 5 mM TG	100	0	0	0	60	20	20
Salt blank, 5mM TG	100	0	60	0	0	0	40
BSA blank, 5 mM TG	100	0	0	60	0	0	40
LPL act., 5 mM TG	100	0	0	0	60	0	40
Salt blank, 2.5 mM TG	50	50	60	0	0	20	20
BSA blank, 2.5 mM TG	50	50	0	60	0	20	20
LPL act., 2.5 mM TG	50	50	0	0	60	20	20
Salt blank, 2.5mM TG	50	50	60	0	0	0	40
BSA blank, 2.5 mM TG	50	50	0	60	0	0	40
LPL act., 5 mM TG	50	50	0	0	60	0	40
FA recov., Salt blank	100*	0	60	0	0	40	0
FA recov., BSA blank	100*	0	0	60	0	40	0
FA recov., LPL act.	100*	0	0	0	60	40	0

* FA premix

Appendix B: Description of publications used to generate the amino acid database described in chapter 4.

Diet[1]	CP in Diet (% DM)	DIM[2]	DMI (kg/d)	Infusate/Rate[3] (g/d)	Infusion[4] Site	Milk Yield (kg/d)	True protein Yield[5] (g/d)	Reference
GS, Barley	14.5	137	16.2	------	JV	23.8	771*	Metcalf et al., 1996
			16.7	TAA/400		24.4	854*	
			16.2	------		22.4	728*	
			16.7	EAA/208		23.5	867*	
AH, Corn, SBM	16.5	220	26.2	------	AB	26.5	832	Mackle et al., 1999b
			27.6	CAS/500 + BCAA/88		27.6	875	
GS, Barley, SBM	14.6	123	13.9	------	AB	17.7	516	Choung and Chamberlain, 1995a
			14.2	CAS/100		17.9	538	
			14.4	CAS/200		18.8	588	
			14.4	CAS/400		19.5	609	
			13.9	CH/110		17.7	520	
			14.1	CH/220		17.0	534	
			14.2	CH/440		18.6	587	
GS, Barley, SBM	16.6	123	13.6	------	AB	18.2	510	Choung and Chamberlain, 1995a
			14.2	CAS/160		19.5	570	
			13.4	CAS/320		20.3	618	
			13.7	TAA/146		18.8	535	
			13.6	TAA/292		20.2	615	
GS, Barley, FM	19.3	74	13.8	------	JV	17.4	487	Choung and Chamberlain, 1995b
			14.1	His/9.7, Met/9.1,Lys/30, Trp/2.6		20.0	615	
			13.6	His/9.7, Met/9.1,Lys/30, Trp/2.6		18.6	541	

Diet[1]	CP in Diet (% DM)	DIM[2]	DMI (kg/d)	Infusate/Rate[3] (g/d)	Infusion[4] Site	Milk Yield (kg/d)	True protein Yield[3] (g/d)	Reference
GS, Barley, FM	17.3	74	13.6	------	JV	17.1	424	Choung and Chamberlain, 1995b
			13.7	His/7.0, Met/8.3,Lys/20.5, Trp/4.0		18.8	503	
			14.0	His/7.0, Lys/20.5, Trp/4.0		19.3	499	
TS, CS, Corn	14.4	207	23.8	------	AB	36.9	1107	Robinson et al., 2000
			23.1	Lys/50		35.8	1051	
			21.9	Met/15.9		34.2	1042	
			22.0	Lys/49.4, Met/16.2		34.5	1042	
TS, CS, barley	14.6	50	21.4	------	AB	33.6	902	Robinson et al., 1999
			21.3	Ile/30.7		35.1	921	
GS, barley	14.1	140	13.6	------	AB	13.9	408	Choung and Chamberlain, 1993a
			13.7	CAS/110		15.7	436	
			13.7	CAS/220		17.1	510	
			14.3	CAS/330		17.4	557	
CS, corn, SBM	14.3	133	17.9	------	AB	23.3	698	Seymour et al., 1990
			17.1	CAS/400		24.4	744	
			17.2	CH/400		23.6	698	
			16.8	Lys/30.1, Met/11.3		22.9	707	
CS, corn, SBM, CGM	14.3	133	17.1	------	AB	23.4	670	Seymour et al., 1990
			17.2	CAS/400		24.6	716	
			17.4	CH/400		24.3	716	
			16.8	Lys/30.1, Met/11.3		23.5	688	
CS, GNM, SBM	16.3	89	15.3	------	DU	24.1	660*	Guinard et al., 1994
			15.3	CAS/177		25.5	717*	
			15.2	CAS/352		25.3	718*	
			15.3	CAS/762		26.7	786*	

Diet[1]	CP in Diet (% DM)	DIM[2]	DMI (kg/d)	Infusate/Rate[3] (g/d)	Infusion[4] Site	Milk Yield (kg/d)	True protein Yield[5] (g/d)	Reference
AH, Corn, SBM	16.6	184	20.0	------	AB	26.3	761*	Griinari et al., 1997a, 1997b
			19.6	CAS/500		28.6	831*	
AH, Corn, SBM	16.2	112	24.8	------	AB	32.3	930*	Mackle et al., 1999a
			24.3	BCAA/150		32.5	920*	
			23.7	CAS/600		33.2	976*	
			24.1	BCAA/44, CAS/600		33.4	979*	
CS, GNM, SBM	17.8	119	17.0	------	DU	24.2	674*	Guinard and Rulquin, 1995
			17.1	Met/8		23.2	660*	
			17.1	Met/16		24.3	707*	
			17.0	Met/32		24.0	689*	
GS, Barley, SBM	15.3	126	13.8	------	AB	20.2	582	Choung and Chamberlain, 1992b
			14.4	CAS/230		22.4	674	
CS, AS, Corn	16.0	28	20.4	Met/12	DU	37.4	921	Schwab et al., 1992a
			21.4	Lys/30		39.8	1010	
			21.9	Met/12, Lys/30		40.0	1056	
			20.3	CAS/400		40.0	1054	
CS, AS, Corn	16.0	70	21.1	Met/12	DU	35.1	877	Schwab et al., 1992a
			21.5	Lys/30		35.6	923	
			21.6	Met/12, Lys/30		35.0	925	
			21.9	CAS/400		37.0	980	
CS, AS, Corn	16.0	133	21.2	Met/10	DU	28.8	766	Schwab et al., 1992a
			21.3	Lys/25		29.3	777	
			21.9	Met/10, Lys/25		29.8	818	
			21.8	CAS/333		30.9	856	

Diet[1]	CP in Diet (% DM)	DIM[2]	DMI (kg/d)	Infusate/Rate[3] (g/d)	Infusion[4] Site	Milk Yield (kg/d)	True protein Yield[5] (g/d)	Reference
CS, AS, Corn	16.0	203	18.6	Met/8	DU	21.1	584	Schwab et al., 1992a
			18.4	Lys/20		20.9	584	
			19.2	Met/8, Lys/20		21.2	617	
			17.8	CAS/266		20.8	588	
CS, AS, Corn	17.3	28	17.3	------	DU	31.7	781	Schwab et al., 1992b
			17.8	Met/10, Lys/10		32.2	908	
			18.8	Met/10, Lys/20		34.7	966	
			18.8	Met/10, Lys/30		35.5	995	
CS, AS, Corn	14.3	105	20.4	------	DU	32.5	853	Schwab et al., 1992b
			20.3	Met/10, Lys/10		32.4	870	
			21.5	Met/10, Lys/20		33.1	910	
			20.8	Met/10, Lys/30		32.8	894	
CS, AS, Corn	14.6	154	20.7	------	DU	30.4	836	Schwab et al., 1992b
			20.4	Met/10, Lys/10		31.4	886	
			20.9	Met/10, Lys/20		30.4	880	
			20.6	Met/10, Lys/30		30.7	895	
CS, AS, Corn	14.8	210	18.7	------	DU	23.0	671	Schwab et al., 1992b
			18.2	Met/8, Lys/8		20.7	622	
			19.3	Met/8, Lys/16		22.1	682	
			18.5	Met/8, Lys/24		22.1	678	
GH, WS, Barley	11.0	7	14.3	------	AB	22.1	565	König et al., 1984
			14.3	CAS/240		23.8	648	
			14.3	CAS/460		23.5	635	
GS, Barley	14.8	88	14.1	CAS/230	AB	20.1	563	Choung and Chamberlain, 1993b
			13.8	CH/260		20.2	562	

Diet[1]	CP in Diet (% DM)	DIM[2]	DMI (kg/d)	Infusate/Rate[3] (g/d)	Infusion[4] Site	Milk Yield (kg/d)	True protein Yield[5] (g/d)	Reference
CS, GH, Corn	15.2	94	19.9	------	DU	29.1	771	Lynch et al., 1991
			19.4	Lys/24, Met/8		27.9	744	
CS, AHL, Corn	15.0	82	18.7	------	AB	25.4	716	Cohick et al., 1986
			19.0	CAS/395		27.2	800	
			12.3	------		21.5	577	
			12.7	CAS/395		24.0	688	
CS, GNM, SBM	17.7	144	17.0	------	DU	21.7	670*	Guinard and Rulquin, 1994
			16.6	Lys/9		22.4	702*	
			16.7	Lys/27		21.8	698*	
			17.0	Lys/63		21.8	683*	
CS, SBM, Corn	17.6	35	23.0	------	DU	37.5	1020*	Pisulewski et al., 1996
			22.2	Met/6		37.9	1046*	
			22.6	Met/12		36.3	1038*	
			23.1	Met/18		36.6	1076*	
			22.9	Met/24		37.1	1102*	
CS, Corn, SBM	16.8	61	20.0	------	DU	31.5	856	Aldrich et al., 1993
			19.8	Lys/24, Met/8		32.8	893	
AH, Barley, Corn	14.9	102	16.9	CAS/400	AB	23.9	718	Cant et al., 1991
	15.9		17.4	CAS/400		27.0	753	
GS, Barley, Oats	14.2	110	19.1	------	AB	24.9	743*	Varvikko et al., 1999
			19.0	Met/10		24.7	732*	
			19.6	Met/20		25.3	763*	
			19.8	Met/30		25.6	768*	
			19.2	Met/40		25.3	759*	

Diet[1]	CP in Diet (% DM)	DIM[2]	DMI (kg/d)	Infusate/Rate[3] (g/d)	Infusion[4] Site	Milk Yield (kg/d)	True protein Yield[5] (g/d)	Reference
GS, Barley, Oats	13.5	196	17.4	------	AB	22.0	663*	Varvikko et al., 1999
			17.8	Lys/15		22.1	672*	
			17.8	Lys/30		22.2	670*	
			17.7	Lys/45		21.9	665*	
			17.6	Lys/60		22.0	654*	
GS, Barley, Oats	13.4	105	16.1	------	AB	22.9	646	Vanhatalo et al., 1999
			16.3	His/6.5		23.6	671	
			16.3	His/6.5, Met/6		23.7	677	
			16.2	His/6.5, Lys/19		24.2	667	
			16.4	His/6.5, Met/6, Lys/19		23.7	678	
CS, Corn, CGM	15.7	49	19.2	------	AB	26.4	763	King et al., 1991
			19.2	Lys/45		27.4	818	
			19.5	Lys/90		27.7	884	
CS, Corn, CGM	15.7	35	23.1	------	AB	29.7	874	King et al., 1991
			22.7	Lys/22.5		31.2	939	
			23.0	Lys/45		30.1	911	
			22.7	Lys/90		31.2	949	
			22.7	Lys/180		32.1	967	
GS, Barley	13.7	98	12.2	------	JV	15.9	447	Chamerlain and Thomas, 1982
			11.6	Met/8		15.7	426	
GS, Barley, SBM	14.7	182	14.3	------	AB	16.9	498	Choung and Chamberlain, 1992a
			14.9	CAS/230		19.5	581	
GS, Barley, SBM	14.6	56	15.4	------	JV	22.4	620	Choung and Chamberlain, 1992a
			15.1	Met/5, Phe/9.1, Trp/2.2		21.9	616	
	18.7		16.0	------		23.0	671	

Diet[1]	CP in Diet (% DM)	DIM[2]	DMI (kg/d)	Infusate/Rate[3] (g/d)	Infusion[4] Site	Milk Yield (kg/d)	True protein Yield[5] (g/d)	Reference
GS, Barley, SBM	14.6	56	15.4	------	JV	22.4	620	Choung and Chamberlain, 1992a
			15.1	Met/5, Phe/9.1, Trp/2.2		21.9	616	
	18.7		16.0	------		23.0	671	
			15.7	Met/5, Phe/9.1, Trp/2.2		23.0	664	
BS, BP	13.6	20	9.1	------	AB	13.0	361	Whitelaw et al., 1986
			9.4	CAS/200		15.3	436	
			9.7	CAS/400		16.5	479	
			9.7	CAS/600		17.2	508	
AH, Corn	18.0	--	20.5	------	AB	30.6	970	Rogers et al., 1984
			20.4	CAS/462		32.7	1066	
GS, Barley, FM	20.0	154	17.8	------	JV	14.2	482	Kim et al., 2000
			16.9	EAA/114.4		17.1	581	
			17.3	His/6, Met/7.1, Lys/17.6		16.6	582	
			16.9	His/6		16.5	538	
GS, Barley, FM	21.2	39	18.1	------	JV	27.0	717	Kim et al., 2000
			18.2	His/9, Met/10, Lys/25.5, Trp/4.8		27.8	811	
			18.6	His/9, Lys/25.5, Trp/4.8		27.5	729	
			18.5	His/9, Met/10, Trp/4.8		27.2	755	
			19.1	His/9, Met/10, Lys/25.5		28.6	834	
GS, Barley, FM	21.1	42	16.4	------	JV	25.9	688	Kim et al., 1999
			16.7	His/9.7, Met/9.1, Lys/30, Trp/2.6		28.4	813	
			16.1	Met/9.1, Lys/30, Trp/2.6		25.2	665	
			16.7	His/9.7, Met/9.1, Trp/2.6		28.0	791	
GS, Barley, SBM	14.7	70	14.2	------	AB	14.4	432	Choung and Chamberlain, 1992c
			14.9	CAS/230		16.6	519	

Diet[1]	CP in Diet (% DM)	DIM[2]	DMI (kg/d)	Infusate/Rate[3] (g/d)	Infusion[4] Site	Milk Yield (kg/d)	True protein Yield[5] (g/d)	Reference
GS, Barley	13.7	56	18.4	------	DU	25.2	769	Huhtanen et al., 1997
			17.4	CAS/400		24.9	734	
			17.9	------		27.4	854	
			17.9	CAS/400		26.7	829	
BS, Barley	13.1	7	11.2	------	AB	16.8	476	Ørskov et al., 1977
			11.0	CAS/300		19.5	601	
GH, Corn	11.5	70	16.3	------	AB	23.3	616	Schwab et al., 1976
			17.2	Met/7.1		23.0	604	
			17.0	Met/7.1, Lys/22.1		23.0	640	
			17.4	Met/7.1, Lys/22.1, Val/18.1		23.6	646	
			18.3	Met/7.1, Lys/22.1, Val/18.1, Ile/16.1		23.8	669	
			17.7	EAA/97.3		23.9	655	
			18.1	EAA/123.3		24.9	697	
			17.7	EAA/136.6		24.6	696	
GH, Corn	10.7	70	17.9	------	AB	29.1	738	Schwab et al., 1976
			16.9	EAA/119.8		29.8	793	
			17.6	EAA/164.8		29.4	815	
			17.7	CAS/425		30.8	830	
CS, Corn	10.7	70	19.1	------	AB	27.2	708	Schwab et al., 1976
			19.3	Lys/27.8, Met/11.3		27.1	747	
			19.2	Lys/27.8, Met/11.3, Val/25		26.4	741	
			19.3	Lys/27.8, Met/11.3, Val/25, Phe/19.6, Ile/22.3		27.0	751	
			19.4	CAS/425		27.7	777	

Diet[1]	CP in Diet (% DM)	DIM[2]	DMI (kg/d)	Infusate/Rate[3] (g/d)	Infusion[4] Site	Milk Yield (kg/d)	True protein Yield[5] (g/d)	Reference
CS, Corn	10.9	70	20.5	------	AB	28.4	787	Schwab et al., 1976
			20.5	Lys/27.8		28.9	804	
			20.2	Lys/27.8, Met/11.3		29.2	829	
			19.9	Lys/27.8, Met/11.3, Val/25		28.7	818	
			20.5	CAS/425		29.8	864	
CS, Corn	10.7	70	18.9	------	AB	29.4	791	Schwab et al., 1976
			18.3	Lys/27.8		29.7	801	
			18.1	Lys/27.8, Met/11.3		29.8	829	
			19.2	Lys/27.8, Met/11.3, Thr/15.2		31.1	860	
			19.1	His/10.2, Arg/13.7		30.7	813	
			19.6	CAS/425		32.2	910	
AGH, Corn	17.7	--	18.5	------	AB	28.8	896	Clark et al., 1977
			18.7	CAS/450		31.0	997	
AGHL, CS, Corn	15.3	80	20.5	------		30.7	874	Vicini et al., 1988
			21.0	Arg/109	JV	31.9	846	
			20.2	Arg/178	AB	33.4	865	
CS, Corn, Barley, SBM	14.0	34	13.9	------	DU	35.0	911	Bruckental et al., 1991
			14.5	Pro/80		36.0	920	
	14.0	121	13.8	------	DU	23.6	607	
			14.7	Pro/80		24.9	693	
GS, Barley, SBM	13.8	210	12.7	------	AB	12.1	417*	Choung and Chamberlain, 1995c
			13.1	CAS/180		13.1	473*	
GS, Barley, FM	19.0	70	11.0	------	AB	10.7	318*	Choung and Chamberlain, 1995c
			11.3	CAS/230		13.6	416*	

[1]Primary ingredients in ration; AGH = alfalfa-grass hay, AGHL = alfalfa-grass haylage, AH = alfalfa hay, AHL = alfalfa haylage, AS = alfalfa silage, BP = beet pulp, BS = barley straw, CGM = corn gluten meal, CS = corn silage, FM = feather meal, GH = grass hay, GNM = groundnut meal, GS = grass silage, SBM = soybean meal, TS = timothy silage, WS = wheat straw

[2]Days in milk at start of experiment

[3]CAS = casein, CH = casein hydrolysate, EAA = essential AA, NEAA = nonessential AA, TAA = EAA + NEAA

[4]AB = abomasal, DU = duodenal, JV = jugular vein

[5]Calculated as published crude protein yield × 0.93 (NRC, 2001) except where indicated by *

*Published true protein yield